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October-December, 1959 (Inclusive)

VOLUME 102

New York

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Printed by
THOMAS J. GRIFFITHS SONS, INC.
Utica, N. Y.

Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 102 OCTOBER 1959 No. 1

SECTION MEETINGS

SOUTHERN CALIFORNIA
University of Southern California June 4, 1959
San Diego State College June 16, 1959

Isolation from Cattle of a Virus Related to Human Adenovirus.* (25123)

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(Introduced by M. J. Oppenheimer)
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Recent studies of inhibitors in bovine sera to certain human viruses—polioviruses, Coxsackie, and adenoviruses(1-5) have raised the question of the nature and origin of these inhibitors. We concluded, on the basis of previous studies on the nature of these neutralizing substances to types 1, 2, and 3 polioviruses, that they were indeed true antibodies. This observation indicated that cattle were perhaps infected either with these viruses or their antigenic relatives. We accordingly undertook a search for these presumed agents, and the present report describes identification of one virus among some 70 viral isolates obtained from feces of apparently normal cattle. This bovine virus, which we originally detected by screening procedure with human gamma globulin(3), has been identified as an adenovirus related to some as yet unidentified human adenovirus type.

Materials and methods. Fecal swabs were obtained from cattle in the Philadelphia area and immediately placed in tubes of broth containing 500 units of penicillin, 500 units of streptomycin, and 1,000 units of nystatin. Tubes were centrifuged at 3,000 rpm for 20 minutes. The supernatant fluid was removed and incubated at 37°C for 45 minutes to further reduce contaminants. Contamination was only infrequently encountered. The tubes were either stored at –70°C or inoculated directly into tissue culture tubes. These were prepared from freshly trypsinized calf kidney. Growth medium initially used was 80% Eagle's medium in Hanks' BSS plus 20% horse serum. Lactalbumin hydrolysate (0.5%) plus 10% horse serum was subsequently used as growth medium. Maintenance medium was 78% Eagle's medium in Earle's BSS, 20% trypticase soy broth plus 2% chick serum. Calf serum was not used since we wished to avoid any inhibitors to bovine viruses. Several commercial lots of Merck, Sharp & Dohme human gamma globulin were used. Since there was some concern about the inhibitory action of preservatives in the material, special lots of gamma globulin were prepared without preservative from material kindly sent us by Dr. Singher of Ortho Research Foundation. Wassermann negative sera obtained from the hospital serology laboratory. Sera were obtained from children in

* Supported by U. S. Public Health Service Grant.

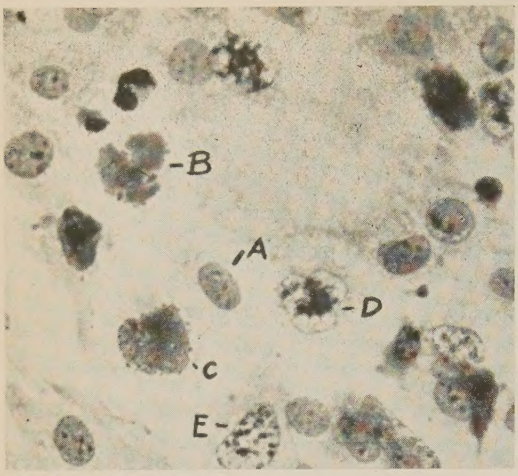

FIG. 1. Bovine #10 grown in calf testes, 3 days, May-Greenwald-Giemsa. A. Normal nucleus. B, C, D, E, enlarged nuclei with varying patterns of chromatin clumping.

age group 7-15 months. Antisera to adenoviruses types 1-7 were kindly given us by Dr. Bartell of Wyeth Institute. Antisera to ECHO viruses types 1-19; Coxsackie virus B types 1-5; antisera to viruses of herpes simplex, measles, mumps, B virus; influenza A, B, swine, Asian; hemadsorption virus types 1 and 2 were kindly supplied by Drs. Hummeler and Lief of Children's Hospital, Philadelphia. Antisera to types 1, 2, and 3 polioviruses were obtained from National Foundation. Complement fixation tests confirming Bovine #10 as an adenovirus were kindly carried out by Dr. Hummeler of Children's Hospital.

Results. The virus Bovine #10 was isolated from feces of apparently normal adult cow in May, 1956. The original cytopathogenic effect in freshly trypsinized calf tissue culture was slight and after 7 passages in calf kidney tissue culture the titer has remained low, never higher than 10^{-3} (0.1 ml) of inoculum. The virus gives a characteristic intranuclear inclusion body when stained with May-Greenwald-Giemsa (Fig. 1). The nucleus is enlarged, there is clumping of chromatin either in large sheets or irregular masses, and the entire sequence of events is similar to that described for human adenoviruses types 3, 4, and 7(6). The growth properties of Bovine #10 are as follows: No detectable activity in: 1. Mice (adult and suckling); 2. Chick embryos (chorio-allantoic membrane, allantoic cavity, amniotic cavity); 3. Guinea pigs, rabbits, hamsters; 4. Hemagglutination and hemadsorption tests (red cells from guinea pig, sheep, cow, chicken, human 'O'); 5. HeLa cells. The only identifying properties of Bovine #10 were a characteristic intranuclear inclusion body and growth at a low titer, 10^{-2} to 10^{-3} in cell cultures of calf kidney and calf testes. There was only a slight cytopathogenic effect in monkey kidney tissue culture and we have had considerable difficulty in passaging the agent in monkey kidney tissue culture. We did not obtain growth in HeLa cells, though 3 passages were carried out.

We concluded that Bovine #10 was related to a human agent because (a) it was neutralized by human gamma globulin; and (b) neutralized by significant percentage of individual adult sera and (c) it was not neutralized by sera of infants. In Table I results of neutralization tests with human gamma globulin are recorded. A 1:160 dilution of human gamma globulin neutralized 100 tissue culture doses of Bovine #10, though there is marked variation with the challenge dose. This level of neutralization is highly significant. In Table II are recorded results of neutralization tests of Bovine #10 with sera of children and adults. Thirty-eight % of 50 adult sera neu-

TABLE I. Neutralization of Bovine Virus #10 by Human Gamma Globulin.

Dilution of commercial globulin	Virus challenge TCD 10	100	1000
1:20	Neut.	Neut.	Neut.
1:40	"	"	Neg.
1:80	"	"	"
1:160	"	"	"
1:320	"	Neg.	"

TABLE II. Neutralization of Bovine Virus #10 by Sera of Adults and Infants.

No. tested	Neg.(1:8)	Positive 1:8	1:16	1:32	1:64	% positive
		Adult sera *vs* 100 TCD				
50	31 (62%)	9	7	2	1	38%
		Infants' sera *vs* 10 TCD				
25	25		—			0%

Age of infants ranged 7-15 mo.
All sera heated to 56°C for 30 min.

TABLE III. Incidence of Neutralizing Antibodies in Adult Bovine Sera to Bovine Virus #10.

No. tested	Serum dilution 1:32+	Serum dilution 1:8		Total positive
40				35(87.5%)
	Pos.	Pos.	Neg.	
	21(52.5%)	14(35%)	5(12.5%)	

tralized the virus in dilutions ranging 1:8 to 1:64 while none of the 25 infant sera neutralized the agent. On the basis of this pattern of neutralization by (a) human gamma globulin (b) individual adult sera, but not (c) infant sera, we may conclude that Bovine #10 is related to a human infectious agent.

Bovine #10 is a bovine virus and not a human laboratory contaminant for the following reasons. (a) It was reisolated from the original fecal specimen. (b) It grows in continuous passage in calf kidney and calf testes but not in HeLa cells. (c) The cow from which the agent was isolated showed a rise in neutralizing antibody titer from zero to 1:16 when sera were collected 6 weeks after isolation of the agent. As shown in Table III, the agent is neutralized by 87% of adult bovine sera. We may conclude that this is a bovine virus, a newly recognized virus, since it is not related to any bovine virus that has yet been described.

In an attempt to identify the specific human relationship, neutralization tests were carried out with antisera against the following viruses, all of which tests were negative. ECHO viruses, types 1-19; Coxsackie B viruses Types 1-5; polioviruses, types 1, 2, and 3; viruses of herpes simplex, measles, mumps, B virus; influenza A, B, swine, Asian; hemadsorption viruses types 1 and 2.

The following experiment established Bovine #10 as an adenovirus. Hamsters and guinea pigs were immunized with Bovine #10 virus grown in calf kidney tissue culture and their antisera tested by the complement fixation test using human adenovirus antigen grown in HeLa cells. There was a certain irregularity in response to the complement fixing antigen of Bovine #10 by the animals. Occasionally the results were obscured by a reaction of the serum with the HeLa cell tissue controls; one of 4 groups of animals immunized with Bovine #10 showed no rise in complement fixing titer against the human adenovirus in spite of a significant neutralizing titer against Bovine #10; typically however the response was clear and the results are shown in Table IV. Neither guinea pigs nor hamsters used in the test had normal inhibitors to adenoviruses before immunization and there was no cross reaction between control tissue in which adenovirus antigen was grown and our immune sera. The results show an unequivocal rise to adenovirus antigen both in antisera prepared in guinea pig and hamster, which identifies Bovine #10 as an adenovirus. Since Bovine #10 is neutralized by human sera and is an adenovirus, it must be an adenovirus belonging either to one of the 18 known human types or to an as yet unidentified human type. We have not yet been able to identify the type of adenovirus, though it is apparently not a type 1-7 based on neutralization tests with antisera against these viruses.

Discussion. Presence of inhibitors in bovine sera to types 1-7 adenoviruses was reported by Gold and Ginsburg(5), though they refrained from interpreting the nature of these inhibitors. We believe our data strongly suggest that if cows are infected with one adenovirus related to human adenoviruses the inhibitors in bovine sera may be considered both as antibodies and indicators of yet other adenoviruses. We have data indicating presence of yet another adenovirus type in cattle.

TABLE IV. Reaction of Antisera to Bovine #10 with Complement Fixing Antigen of Human Adenovirus.

Bovine #10 antisera	C.F. antigen	Complement fixing titer
Guinea pig		
Pre-immunization sera	Human adenovirus	<1:8
	Tissue control*	"
Post-immunization	Human adenovirus	*1:32*
	Tissue control	<1:8
Hamster		
Pre-immunization	Human adenovirus	<1:8
	Tissue control	"
Post- "	Human adenovirus	*1:32*
	Tissue control	1:8

* Guinea pigs and hamsters immunized with virus grown in calf kidney.

C.F. adenovirus antigen grown in HeLa cells (tissue control).

Whether Bovine #10 is identical with a human adenovirus or an antigenic relative remains to be determined. If it is identical with a human virus we may have identified an important reservoir of these agents, for the very high incidence of infection in cattle indicates the agents are endemic in these aninals. We are inclined to believe, however, that Bovine #10 is antigenically related to a human virus, in the sense of a cowpox-smallpox relationship, since we have not been able to achieve one characteristic of human adenoviruses, namely growth in HeLa cells. There is of course a vaccine potential if this latter relationship is the proper one.

We believe that isolation of an adenovirus from cattle gives added weight to the implication, based on neutralizing antibodies to polioviruses in calf sera, that cattle may harbor polioviruses or their antigenic relatives. We may note in passing that 45% of bacterial, fungal, and parasitic infections of animals are shared by man, and if we accept this figure as one that will be valid for viruses we must conclude that a large number of human viruses or their antigenic relatives will ultimately be isolated from animals.

Summary. A virus has been isolated from feces of an apparently normal cow that has been identified as an adenovirus related to or identical with an as yet unidentified type of human adenovirus.

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Received May 6, 1959. P.S.E.B.M., 1959, v102.

Effect of Compound 48/80 on Toxicity of Histamine and of Serotonin in Mice.* (25124)

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Tissue mast cells rapidly respond to various types of injurious stimuli by shedding their cytoplasmic contents (*i.e.,* granules) into the surrounding ground substance of the connective tissue. Since these cells contain relatively large concentrations of both histamine and heparin and, in the mouse and rat, also serotonin (5-hydroxytryptamine)(1,2,3), release of these biologically-active substances undoubtedly influences the nature of subsequent responses of the local tissue to the injurious stimulus. Degranulation of mast cells can be readily initiated by a synthetic phenyl alkylamine substance called compound 48/80 (4). This drug is a potent releaser of histamine(5), has a protamine-like effect on heparin(4) and, in mouse and rat, will also release serotonin(6,7). In addition, it produces a lethal intoxication in the mouse and various other laboratory animals(8). Although Riley (9), and others(5,10), suggested that pharmacologic or injurious effects of compound 48/80 are mediated by the histamine and/or 5-hydroxytryptamine released from mast cells, it would seem unlikely that such a mechanism could account for *lethal* effects of this drug in the mouse. In the first place, tissue levels of histamine and serotonin in the mouse(11) are many times less than those required for lethal effects of these amines in this species (12,13). Secondly, the interaction of compound 48/80 with mast cells should result in

* Research performed during tenure of Lederle Medical Faculty Award (1957-58) and supported by grants from Dept. of Army and Burroughs Wellcome & Co., Tuckahoe, N. Y.

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binding of this drug by heparin with a concomitant reduction in its lethal effects(14). The following report is concerned with the relative importance of mast cell constituents (*i.e.*, histamine, serotonin and heparin) in lethal intoxication produced by compound 48/80 in the mouse.

Materials and methods. CBA mice, mixed sexes, 2 to 3 months of age and ranging in weight from 19 to 21 g were used. Drugs used were: histamine dihydrochloride, histidine chloride, 5-hydroxytryptamine creatinine sulphate and tryptamine chloride obtained from Nutritional Biochemical Corp. Amounts used are given in terms of the active constituent. Compound 48/80‡ was obtained from Burroughs Wellcome & Co., and Na heparinate (117 μ/mg)‡ from Upjohn Co. All drugs were prepared for injection in isotonic, pyrogen-free, sterile saline and administered either singly or in combination in 0.25 ml to the mice *via* tail vein. Intravenous route was used since it is the most sensitive means for detecting lethal effects of histamine, serotonin or compound 48/80. To evaluate toxicity of each of the test drugs, or combinations of drugs, a minimum of 10 animals was employed for each test group and determinations made on the basis of numbers of animals surviving 24 hours after challenge. Results of titrations are presented as 50% lethal doses or 50% protective doses (heparin) as determined by the method of Reed and Muench(15).

Results. Histamine and serotonin. Preliminary experiments were performed by injecting control groups of mice with relatively small doses of either histamine or serotonin or their analogues, histidine or tryptamine, respectively. Comparable groups of test animals were injected with equivalent doses of test substances to each of which had been added a standard sublethal amount of compound 48/80 (2.0 μg/g). Histamine was markedly toxic in presence of compound 48/80, whereas histidine at comparable dose levels was not (Table I). Likewise, the effect of compound 48/80 on toxicity of serotonin was far more marked than on tryptamine. This toxic effect could also be demonstrated if compound 48/80 and amine were given separately but was markedly decreased if time interval between doses was greater than 15 minutes.

TABLE I. Effect of Compound 48/80 on Toxicity of Histamine, 5-OH Tryptamine and Related Substances.

Test drugs (μg/g)	Survival ratios (S/T)* Saline controls	Plus compound 48/80 (2 μg/g)
Saline		10/10
Histamine (60)	10/10	1/10
" (80)	"	10/10
5-OH tryptamine (1)	"	0/10
Tryptamine (4)	"	9/10

* Groups of mice challenged by intrav. route with 0.25 ml saline containing test drug alone or in combination with standard sublethal dose of compound 48/80.

A dose relationship was determined between compound 48/80 and the amines, histamine and serotonin. A series of titrations were performed in each of which groups of mice were challenged with graded doses of one of the amines to which had been added one of a number of standard sublethal amounts of compound 48/80 (0, 1, 1.5 or 2 μg/g). Amount of amine required for a lethal dose effect was determined for each compound 48/80 dose levels. Results are shown in Fig. 1 in which each standard sublethal dose of compound 48/80 (abscissa) are plotted against respective complementary amount of amine (free base) required for an LD_{50} effect. With histamine, the points approximate a straight line drawn between LD_{50} doses of histamine and of compound 48/80. This would suggest that lethal effects of compound 48/80 and histamine are additive. The compound 48/80-serotonin relationship proved to be somewhat different with remarkably small amounts of serotonin being required for LD_{50} effect in compound 48/80 dose range of 1 to 2 μg/g. This would indicate that compound 48/80 synergizes serotonin toxicity.

Results were 166, 103, 56.6, 26.5 and .0 μg histamine base/g body wt. and 137.4, 26.5, 4.2, .4 and .0 μg serotonin base/g body wt. for LD_{50} effects at compound 48/80 dose levels of .0, 1, 1.5, 2 and 2.2 μg/g, respectively.

‡ Author expresses appreciation to Dr. S. T. Bloomfield of Burroughs Wellcome & Co. and Dr. L. L. Coleman of Upjohn Co. for generous gifts of compound 48/80 and heparin, respectively.

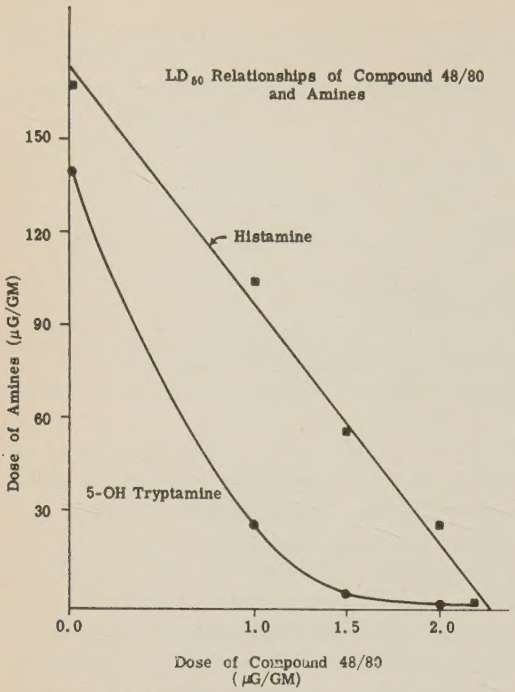

FIG. 1. LD_{50} relationships of compound 48/80 with histamine or 5-hydroxytryptamine in mice. Individual LD_{50} results obtained following intrav. challenge of groups of mice (10 animals in each) with specific doses of compound 48/80 (abscissa) containing graded amounts of one of the amines. Individual LD_{50} doses of either histamine or 5-hydroxytryptamine are many times greater than that of compound 48/80.

When these data are compared to tissue levels of histamine (8 - 10 μg/g)(12,11) and of serotonin (.2 - 2 μg/g)(16,11) reported for the mouse, it becomes evident that the apparent additive or synergistic effects of compound 48/80 on amine toxicity could not be directly due to supplementation of the exogenously administered amines by those released from the tissues. In this regard, it has been observed that serotonin (70 μg/g) did not elevate the sublethal effect of histamine (60 μg/g) to a lethal dose. The lethal effects of compound 48/80 therefore appear to result from a primary toxic effect on tissues which are not solely dependent on release of these amines. The markedly reduced tolerance of mice for either histamine or serotonin in presence of compound 48/80 is considered as evidence of this tissue injury.

Heparin. It has been previously suggested that release of heparin from mast cells in their response to compound 48/80 can be of salutary value in that this acidic mucopolysaccharide will protect against the lethal effects of this cationic drug(14). The relative value of the protective effect of heparin versus compound 48/80-enhancing effect on amine toxicity was investigated. A series of titrations were performed with heparin *versus* standard minimal (100%) lethal doses of histamine, serotonin, mixtures of compound 48/80 with the respective amines or compound 48/80 alone. This was performed by injecting groups of mice with one of the lethal challenge doses to which had been added one of a series of graded amounts of heparin. In this way, 50% protective dose of heparin was determined for each group in the series of challenges shown in Table II.

Heparin, in doses to 200 μg/g, gave no evidence of protection against either histamine or serotonin and only partial protection against 1 μg/g compound 48/80 plus 135 μg/g histamine. Protection in this latter group varied between 30 and 40% with doses of heparin in the range of 50 to 200 μg/g. However, in all other groups only relatively small amounts of heparin were required for complete protection against lethal doses of compound 48/80-histamine, compound 48/80-serotonin or compound 48/80 alone. Heparin protection appeared related to compound 48/80 content of the challenge dose with the ratios of 50% protective doses of heparin to their respective compound 48/80 doses being somewhat variable but less than 1 in the histamine group and approximating 0.2 in the serotonin group. It thus appears that direct interaction of heparin with compound 48/80 can indirectly affect toxicity of the respective amines.

Lethal effects of compound 48/80-amine mixtures are dependent on relative amounts of both constituents (Fig. 1). In each instance of heparin protection versus compound 48/80-enhanced intoxication with either histamine or serotonin, the amount of heparin required was much less than the amount of amine employed (Table II). It was also observed that with a given dose of compound 48/80, the amount of heparin required varied directly with amount of amine involved. If

TABLE II. Effect of Heparin on Compound 48/80-Enhanced Toxicity of Histamine and 5-Hydroxytryptamine.

Compound 48/80 (μg/g)	A. Histamine			B. 5-OH tryptamine		
	Dose (μg/g)	Heparin PD_{50} (μg/g)	Heparin: Cmpd. 48/80	Dose (μg/g)	Heparin PD_{50} (μg/g)	Heparin: Cmpd. 48/80
.0	377.0	200.0		160.0	200.0	
1.0	135.0	50.0*		34.3	.22	.22
1.5	90.0	1.3	.87	10.1	.21	.14
2.0	30.0	.35	.18	.92	.4	.2
2.67	.0	.7	.27	.0	.7	.27

Groups of at least 10 mice each were challenged by intrav. route with 0.25 ml saline containing one of standard mixtures (minimal lethal dose) of compound 48/80 with either (A) histamine or (B) 5-hydroxytryptamine plus one of a series of graded amounts of heparin. Results are presented in terms of 50% protective dose of heparin (PD_{50}). The ratios of these heparin doses to appropriate compound 48/80 doses are also shown.

* % survival did not exceed 40% in heparin dose range of 50 to 200 μg/g.

these relationships could be considered valid for interaction of compound 48/80 with the mast cell, then the contribution of the mast cell to the lethal effects of a given dose of compound 48/80 would depend on the relative amounts of histamine and/or serotonin to heparin content of this cell. Since the literature(17) shows the mast cell to have a smaller amine content (approx. 1% histamine and possibly 0.1% serotonin) than it does heparin (approx. 3%), the exclusive interaction of a given dose of compound 48/80 with mast cells would be more likely to inhibit than to mediate the lethal effects produced by this drug.

Discussion. The biological significance of mast cell degranulation in response to compound 48/80 has been generally related to *release* of histamine and, in some species, of serotonin and to subsequent pharmacologic effects of these amines(5,7,9,10). Although neither histamine nor serotonin is present in tissues of the mouse in sufficient amounts to account directly for the lethal effects of compound 48/80, it was observed that compound 48/80 could markedly increase the lethal effects of these amines in the mouse. Compound 48/80 would thus appear to have a primary lethal effect quite distinct from alterations in the physiologic state of tissues which are induced by the released amines. This point is well supported by the observations of others(8,18,19,20). Since this injurious effect of compound 48/80 can be manifested as a decreased resistance to histamine and to serotonin, the release of these amines may be contributory to the total lethal effect but would not otherwise appear to be of major importance in this regard. Moreover, it has been observed in studies to be reported later, that prior treatment of mice with multiple sublethal doses of either compound 48/80 or polymyxin B to "deplete" their tissues of histamine and serotonin, does not provide any greater degree of resistance to the lethal effects of an *i.v.* challenge with compound 48/80 than can be obtained simply by pretreatment of animals with saline.

As discussed elsewhere(11,21), release of histamine by compound 48/80 is probably the result of an ion exchange in which heparin, serving as a cation exchanger, releases histamine in exchange for compound 48/80. This would result in a reduction in amount of compound 48/80 available to interact at more vital sites in the tissues. Since this drug is many times more toxic than is histamine (or serotonin), this exchange reaction can be of marked benefit to the organism. In this regard, heparin treatment will readily protect mice against lethal effects of compound 48/80 (14). The reaction of mast cell to compound 48/80 has been considered as essentially a protective response of connective tissue to injury and the associated release of histamine and serotonin as primarily a manifestation of this response. The degree to which the released amines may then participate in the lethal effects of this drug would be conditioned by amount of injury directly produced by compound 48/80.

Summary. (1) Compound 48/80 appears to have an additive effect on toxicity of hista-

mine and a synergistic effect on toxicity of serotonin in the mouse. (2) Heparin interferes with this compound 48/80 effect on lethal intoxication with histamine or serotonin by direct interaction with compound 48/80 but does not appear to alter individual toxicities of respective amines. (3) It is suggested that degranulation of mast cells in response to compound 48/80 is a protective function of connective tissue in which the releaser drug is sequestered by heparin of the mast cell. The associated release of amines is thought to be a manifestation of this interaction between mast cells and compound 48/80 and only indirectly related (if at all) to lethal effects of this drug.

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Received May 25, 1959. P.S.E.B.M., 1959, v102.

Rapid Sensitive Method for Determining H^3-Water in Body Fluids by Liquid Scintillation Spectrometry.* (25125)

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The assay of tritium water by liquid scintillation spectrometry is difficult for 2 main reasons: a) water is not efficient in transferring the excitation energy of tritium radiation to the phosphor; and b) water is insoluble in the known aromatic solvents (toluene and xylene) which are most effective in making this transfer. In the methods of Kinard (1), Hayes and Gould(2), and Okita *et al.* (3), alcohol is used to solubilize water with xylene or toluene. This results in considerable dilution of the H^3 in the water sample, thus limiting these methods to samples of tritium water with high radioactivity. Furst *et al.* (4) were the first to report that naphthalene can restore, to a large extent, the counting efficiency lost by addition of water to dioxane solutions containing tritium. This observation formed the basis of the more efficient assay developed by Langham *et al.*(5). A method for increasing still further the efficiency of counting tritium water in biological fluids is described here. Some of the conditions that influence the efficiency of the assay of tritiated water by the dioxane-naphthalene

* Aided by grant from U.S.P.H.S.

system have been studied. It is shown that the sensitivity of H^3-water measurements in biological fluids can be greatly augmented by a preliminary distillation of the water in these fluids with benzene. The method evolved is simple in operation, and does away with the need for internal standards(6). It is capable of detecting about 5.7×10^{-5} μc of H^3-water in almost all tissues.

Experimental. A Packard Tri-Carb Model 314 spectrometer was used for H^3 determinations; counting was carried out with a discriminator window setting of 10-90, a voltage of 1340 volts, and a deep-freeze setting of + 3°C. Water was removed from reagent grade benzene by distilling it until the distillate became clear. The benzene remaining in the distilling flask was dry enough for the present experiments. The dioxane and naphthalene were Eastman Kodak products (catalogue Nos. 2114 and 168, respectively), and they were used without further purification. 2,5-diphenyloxazole (PPO), 1,4,-bis-2(5-phenyloxazolyl)-benzene (POPOP), and α-naphthylphenyloxazole (NPO) were purchased from Arapahoe Chemical Inc., and were of scintillation grade. The H^3-water was obtained from the National Bureau of Standards.

Results. Conditions affecting H^3-counting efficiency of dioxane solution containing various amounts of H^3-water. Optimum conditions for liquid scintillation counting of 1-, 2-, and 3-ml samples of water, each containing the same amount of H^3 and each made up to a total volume of 15 ml with dioxane (6.6, 13.6, and 20% of H^3-water in dioxane, respectively), were determined by studying the effects of varying concentrations of naphthalene, PPO, POPOP, and NPO on the counting rates. The results are given in Table I. Exp. 1-3 show that, for 20% solutions, the maximal permissible concentration of naphthalene is about 116 g/liter. At higher concentrations the solutions separate into 2 layers. Since little difference was observed in counting rates of Exp. 1 and 2, the conditions of the former were chosen for further study. Increasing the concentration of PPO decreased the counting rate (Exp. 4). Exp. 5-8 indicate that NPO is inferior to POPOP as a secondary phosphor or spectrum shifter(6). The use of higher concentration of POPOP (0.25 g/liter) seems warranted because of higher counting rate in Exp. 8 than in Exp. 6. The most efficient system for counting 20% H^3-water in dioxane is provided by conditions shown in Exp. 8. Exp. 9 and 10 indicate that smaller volumes (10 ml and 5 ml, respectively) of H^3-water can be assayed without loss of counting efficiency, a finding in agreement with results of Davidson and Feigelson (7). However, the conditions of these 2 experiments are not recommended for counting volumes smaller than 3 ml because, as shown in Exp. 11-19, more efficient conditions were found. For example, in Exp. 13 and 19, counting efficiencies of 11 and 15% were found for solutions containing 13.3 and 6.6% of H^3-water, respectively. The conditions used in these 2 experiments are therefore recommended when less than 3 ml of water is available for counting. By reducing the amount of each constituent shown in Exp. 19 by one-third, as little as 333 μl of H^3-water can be counted with an efficiency of 15%. The data of Table I were obtained by using pure H^3-water-dioxane solutions. Similar results were to be expected with water of body fluids provided the water could be isolated in sufficiently pure form. In their studies on the H^3-water turnover in rats, Thompson and Ballou(8) used a Dean-Stark trap for collecting water during distillation of a tissue with benzene. We also found that almost pure H^3-water can be obtained from plasma, blood, and urine by distilling them with dry benzene.

Method for determination of H^3 content of 1-3 ml samples of water distilled from body fluids. Three-ml sample. Fifty ml of dry benzene and the tritiated sample to be analyzed (blood, plasma or urine) were added to a 100-ml, round-bottom flask. A Dean-Stark trap, provided with a calibrated collecting tube, a stopcock, and a condenser fitted with a drying tube, was attached to the flask. The given mixture was refluxed until about 3.5 ml of water had collected in the trap; the water was drained through the stopcock into a stoppered test tube, and kept at room temperature for at least 1 hour (preferably 2) to permit minute amounts of benzene in the distillate to

TABLE I. Effects of Naphthalene, Scintillator (PPO), and Spectrum Shifter (NPO and POPOP) Concentrations on the Counting Efficiency of Dioxane-Tritium Water Solutions.*

Exp. No.	Water (% by vol)	Naphthalene	PPO	NPO	POPOP	Counts/min.
		g/liter				
1	20.0	100	10.0			4,380
2	"	116	"			4,410
3	"	133	"			3,085
4	"	100	16.6			4,065
5	"	"	10.0	.1		4,555
6	"	"	"		.1	5,290
7	"	"	"	.25		4,735
8	"	"	"		.25	5,405
9†	"	66.6	6.6			4,575
10†	"	33.3	3.3			4,550
11	13.3	100	10.0			5,260
12	"	133	"			5,660
13	"	"	"		.25	7,390
14	6.6	100	"			7,080
15	"	133	"			7,415
16	"	166	"			7,710
17	"	200	"			7,900
18	"	233	"			7,690
19	"	200	"		.25	9,665

* Each solution contained 62,640 disintegrations/min. (DPM) of H^3.

† With exception of these 2 experiments, total vol of counting solution was 15 ml. In Exp. 9 the vol was 10 ml, and in Exp. 10, 5 ml.

rise to the surface before being removed with a micropipette.

To a 5-dram vial provided with a screw cap were added 1.5 g of naphthalene, 12 ml of dioxane containing 150 mg of PPO and 3.75 mg of POPOP, and 3 ml of the distilled H^3-water. The vial was closed, shaken vigorously, and placed in the refrigerated unit of the spectrometer. At least 2 hours were allowed to elapse before the H^3 in the sample was counted. A standard sample of H^3-water was treated in the same way; the benzene distillation was omitted because this operation did not affect its counting rate. *One- and 2-ml. samples.* H^3-water was isolated from samples exactly as described above. The conditions of Exp. 19 and 13 were used for counting the 1-ml and 2-ml of H^3-water, respectively.

Accuracy of method. H^3-water was added to 3 urine samples, and tritium activities of the mixtures were determined by employing the conditions of Exp. 8. The results are recorded in Table II. The values were not corrected for salt content of the urine samples. The calculation given in the footnote of Table II should be used with urine samples that con-

TABLE II. Accuracy of Assaying H^3-Water in Urine and Plasma.

Specimen*			Counting conditions given in designated experiment of Table I	No. of determinations	Found	
		DPM/ml			DPM/ml	% recovery (avg)
Urine	1	31,230	8	4	30,780–31,920	101
	2	500	8	4	479–499	98
	3	625	8	4	608–640	100
Plasma	1†	500	8	5	496–503	100
	2	"	13	2	488	98
	3	"	19	2	521 and 515	104

* Specimens prepared by diluting National Bureau of Standards H^3-water to a known volume with urine or plasma.

† Density of water at room temperature was taken as 1, and therefore aqueous volume of 1 ml of plasma was equal to its loss of weight on desiccation. The following equation was used to determine DPM/ml of plasma: $\frac{\text{DPM found} \times \text{loss in wt of 1 ml plasma on desiccation}}{\text{Vol of water taken for assay}}$.

TABLE III. H^3-Water in Urine of Guinea Pigs That Had Received H^3-β-Sitosterol Intravenously. Conditions of Exp. 8 (Table I) were used for counting H^3-water isolated by distillation.

No. of guinea pigs from which urine was collected	Collection time, hr	Vol of urine, ml	H^3/ml of urine (DPM)*
1	27	29	66
3	40	175	46

* See bottom of Table II for the method of calculation.

tain a high proportion of solid materials. A sample of plasma containing 500 DPM/ml of H^3 was prepared. It was distilled with benzene, and the water isolated was assayed for radioactivity (Table II). Recoveries (average) ranged from 98 to 104%. The most sensitive counting conditions, those of Exp. 8, gave an average recovery of 100%.

Application of the procedure. As part of a study on utilization of H^3-β-sitosterol in the guinea pig, it was of interest to ascertain whether H^3-water was excreted in the urine of guinea pigs that had received intravenously about 1.14 μc of the labeled sterol dissolved in guinea pig plasma. The results are shown in Table III. One animal excreted 1910 DPM of tritium/ml of urine in 27 hours, while the average H^3-DPM/ml of urine for 3 animals in a 40-hour period was 2685. Since guinea pig urine contains considerable quantities of solids, the same method of calculation was used as that for the plasma samples. The data given in Table III confirm our other findings showing that administered β-sitosterol is metabolized by the guinea pig(9).

Discussion. The sensitivity of a system for counting H^3-water is given by the product of the percentage of water in the sample being assayed and the efficiency of the counting method. This product has been referred to as the merit(1). In Table IV we compared merit values for our method with those reported in the literature. Since 50 ml of a solution can be used in commercially available scintillation spectrometers, the merit values reported here and elsewhere have been recalculated to take this volume into account. The values for the recalculated or corrected merits are given in the last column of Table IV.

The corrected merit values of our counting systems, as provided by conditions of Exp. 8 and 13 of Table I, are about twice those of the next best system(1).

The method of Langham *et al.*(4) is superior to any so far reported for assay of H^3 content of body fluids by liquid scintillation spectrometry. The method described here has a merit about 5 times that of Langham *et al.* This is the result of our using higher concentrations of naphthalene in the counting solvent, made possible by the isolation of almost pure water from the urine and plasma specimens. Since Langham *et al.* add urine directly to the counting solution, the quenching effects of varying urine samples have to be corrected by an internal standard(3). This is obviated in our procedure because all normal urines yield water of about the same composition after distillation with benzene.

Since the water content of whole blood or plasma can be recovered almost quantitatively by distillation with benzene, our method can be used for determination of the H^3-water in as little as 1 ml of plasma or blood. For very small samples of plasma, the method of Langham *et al.*(4) would appear to be superior.

The new method can be applied to any tis-

TABLE IV. Merit Values for the New Method Compared with Those for Reported Methods.

Method	% water in sample counted (W)	Counting efficiency (E)	Merit (E · W)	Total vol (ml) of sol counted (V)	F = 50/V	Corrected merit (E · W · F)
Hayes and Gould(2)	3.5	2.8	10	30	1.7	16
Okita *et al.*(3)	4.0	3.0	12	50	1.0	50
Kinard(1)	7.0	9.8*	76	14	3.6	269
Langham *et al.*(5)	4.0	9.0†	36	25	2.0	72
Exp. 8, Table I	20.0	8.6	172	15	3.3	570
13, "	13.3	11.8	157	15	3.3	523
19, "	6.6	15.4	102	15	3.3	340

* Determined at 3° on our counter. † Highest efficiency reported by Langham *et al.*(5).

sue from which water can be removed by distillation with benzene. When the method is used for whole blood, plasma, or urine, a determination of the water content of the sample is required. This is readily performed by removing the water from a weighed sample with an infrared lamp and drying the residue to constant weight.

Summary. 1. An accurate and convenient method for determination of tritium water in body fluids by liquid scintillation spectrometry is described. The method is based on distillation of body fluids with benzene to provide an almost pure sample of H^3-water, and use of an effective solvent (dioxane-naphthalene) to transfer the excitation energy of tritium to the phosphors, 2,5-diphenyloxazole and 1,4-bis-2(5-phenyloxazolyl)-benzene. 2. When applied to determination of tritium water in urine and plasma, the method gave recoveries ranging from 98 to 104%. 3. By the new method, metabolism of intravenously administered H^3-β-sitosterol to H^3-water was demonstrated in the guinea pig.

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Received June 22, 1959. P.S.E.B.M., 1959, v102.

Mesenteric Artery Electrolytes in Experimental Hypertension.* (25126)

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Our previous studies were concerned with the possibility of a significant connection between disturbances in tissue electrolytes and hypertensive vascular disease in the rat(1,2). Such a connection is also suggested by the accelerating effect of salt on renal hypertension (2) and by experimental induction of hypertensive vascular disease through salt and/or renal ablation(3,4). This aspect of hypertension was explored further in the present study which deals with content of electrolytes and water in mesenteric arteries of hypertensive rats.

Method. Adult white male rats weighing about 200 g were rendered hypertensive by bilateral renal artery ligation(1). Additional rats, some sham-operated, served as controls. All animals were kept on standard diet of chow and water *ad lib.* Control blood pressures were established by the plethysmographic method and then readings were made at intervals to one week after surgery, when final determination was obtained and all animals sacrificed. Under Nembutal anesthesia the rats were exsanguinated by withdrawing blood from heart with a syringe. Serum was separated from this blood and used to determine sodium, potassium, and chloride. The entire mesentery was removed en bloc. After veins were detached by dissection, the arteries comprising the mesenteric tree were separated from surrounding fat. This was readily accomplished within a few minutes by stroking the fatty mass with moderately rigid nylon brush. The vessels were followed out along their entire course to the bowel wall. When the procedure was complete the main divisions of the superior mesenteric artery and their branches were isolated in the form of fine threads. These were then detached and obtained in one mass for analysis by cutting along mesenteric border of small intestine and

* Supported by grants from U.S.P.H.S. and Cleveland Area Heart Soc.

TABLE I. Electrolyte and Water Content of Mesenteric Arteries.

	Na	K	Na + K	Cl	H_2O
Hypertensive rats					
Mesenteric arteries	54.8 12.5	25.1 5.2	80.1 18.0	23.5 5.2	250.5 54.1
Serum	142.0 2.7	3.9 .7		97.2 2.1	
Control rats					
Mesenteric arteries	44.7 8.4	21.8 4.3	66.0 16.2	18.8 6.1	200.8 48.3
Serum	142.5 1.1	5.0 .6		100.7 2.5	

Values are in meq/100 g of dry fat-free solids and per liter of serum. H_2O in g. Figures below each value are stand. dev.

then across superior mesenteric artery. The vessels showed no gross abnormality. The arteries were analyzed for sodium, potassium, chloride, and total water. The tissues were dried at 105°C, ground, and defatted with mixture of ethyl ether and petroleum ether. The fat-free dry solids were then extracted with 10 cc of normal nitric acid. All procedures were carried out in the original tubes. Sodium and potassium were determined by standard flame photometry. Chloride was determined by Volhard's titration method(5). Data on mesenteric arteries were obtained in 20 hypertensive rats and in 20 control rats.

Results. The animals with renal ligation were in good condition when sacrificed. They were all hypertensive with systolic blood pressures generally in 170-190 mm Hg range. Control rats were normotensive.

Values for sodium, potassium, sodium plus potassium, chloride, and water in the mesenteric arteries are shown in Table I along with levels of serum electrolytes. Combined cation values were derived from the mean of sums of sodium and potassium concentration for each rat.

In comparison with normal rats, the mesenteric arteries of hypertensive animals showed elevation of sodium, potassium, chloride, and total water. The increase was statistically valid in each instance. Sodium plus potassium content, expressed as meq/100 g of dry fat-free solids was also increased to statistically significant degree. However, the combined cations showed no significant change over the normal in terms of concentration in tissue water.

Extracellular sodium was calculated by using chloride space as a measure of extracellular fluid according to the method of Hastings and Eichelberger(6). Values obtained in this way indicated that extracellular sodium accounted for only part of the total rise of this cation, and hence that the intracellular phase contained more sodium. However, it is doubtful that chloride space is an adequate measure of extracellular volume in the mesenteric vessels. Further comment on this point is given below.

Serum sodium, potassium, and chloride in hypertensive rats showed reduction from normal levels. This was statistically valid in each instance.

Discussion. Our technic for obtaining mesenteric arteries eliminated perivascular fat almost completely, but did not remove adventitia of blood vessels. Hence, while the bulk of material taken for electrolyte analysis was probably smooth muscle, there was also a substantial amount of connective tissue. In reference to man, the vessels in the rat correspond to large arteries but they also include smaller arteries, *i.e.* terminal branches of superior mesenteric tree extending to bowel wall, and these would be comparable to vessels about 1 mm diameter in the human. Also there are numerous arterioles in the adventitia of larger mesenteric vessels.

Previous information on electrolyte composition of vessels in hypertensive rats has apparently been limited to the aorta(7). However, this structure is not concerned in the origin of hypertension nor does it participate in characteristic lesions observed so frequently in the more distal vascular bed of hypertensive rats. In contrast, mesenteric arteries of the rat are a relatively early and prime site of such disease. Hence electrolyte alterations here are apt to reflect similar changes of small arteries and arterioles throughout the body.

Our data show that there was accumulation of sodium in the mesenteric arteries of rats with acute renal hypertension. This was accompanied by rise in potassium and chloride and also by expansion of water. The last

was sufficient in magnitude so that total cation concentration in tissue water of the hypertensive rats did not differ significantly from that in control animals.

It was not feasible to partition sodium and potassium into extracellular and intracellular fractions through use of chloride space. Sometimes the extracellular fluid volume calculated on this basis in both experimental and control animals left only a very small fraction of tissue water to be assigned to cell water or even resulted in negative cell water concentration. There is some doubt in general as to whether chloride space can properly be applied to smooth muscle and to structures rich in connective tissue(8). Since the extracellular fluid space of blood vessels analyzed in this study was in question, increase in intracellular sodium and potassium in arteries could not be established even though this is quite probable.

An interesting item is the relatively high standard deviation for mean content of sodium and tissue water in the mesenteric arteries. This holds for both normal and hypertensive animals, especially the latter, where the deviation increases along with rise of mean values for sodium and water. It does not apply to potassium or chloride levels. The variability of sodium and tissue water in mesenteric arteries suggests that they constitute a labile system in this location.

The information on electrolyte composition of tissues in hypertensive animals is relatively scant. Moreover there are differences of opinion in regard to both the nature and significance of the data. In Grollman's study(9) no significant change from the normal was observed in electrolyte composition of various organs, including heart of rats rendered hypertensive through choline deficient diet. Using inulin to measure extracellular volume, Ledingham(10) found no significant change in intracellular concentration of either sodium or potassium in hearts of rats with acute renal hypertension and believed that the increase in total sodium content was explained by expansion of extracellular fluid. Tobian and Binion(7) reported an increase in sodium and potassium content of the aorta in hypertensive rats and, through use of chloride space, calculated that not more than one-third of the increase in sodium concentration resulted from elevation in extracellular sodium. Greene and Sapirstein(11) found a substantial rise in total body sodium of rats with renal hypertension and believed that this could not be explained by expansion of extracellular volume. They postulated either deposition of sodium in bone or an increased amount of sodium in intracellular position.

It appears that changes in electrolytes and water which we observed in mesenteric vessels were a direct consequence of the kidney damage employed to induce hypertension. However, the precise mode of origin is not certain. Although the renal injury was severe, it did not result in significant azotemia. Renal dysfunction, resulting in retention of salt and water, is one possible mode of origin. Others include injurious agents of toxic or pressor nature derived from the injured kidneys and acting on the vessel walls. Our study did not encompass these items. Perhaps the electrolyte changes within the arteries were merely incidental to altered renal function and not causally related to the high blood pressure. An alternative is that they played a role in the origin of hypertension by creating an ionic disequilibrium which enhanced vasomotor tone.

Summary. 1) Mesenteric arteries of rats with acute renal hypertension were analyzed for electrolyte and water content. A technic is described for obtaining these vessels. 2) Arteries showed a significant rise in sodium, potassium, and chloride, as well as expansion of total water. However, total cation concentration in tissue water was not significantly altered. 3) Electrolyte changes may have resulted from renal dysfunction associated with retention of salt and water. Whether they were incidental to the hypertensive state or played a role in its genesis is not established.

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Received February 16, 1959. P.S.E.B.M., 1959, v102.

Intracellular Distribution of Vit. B_{12}-Co^{60} in Liver and Kidney of B_{12} Deficient and Normal Rats.*† (25127)

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Results of microbiological assays of fractions of mouse liver(1) indicated that Vit. B_{12} is concentrated in mitochondria. Kidneys of rats(2) contained most radioactivity 96 hours after parenteral administration of B_{12}-Co^{60}. At 48 hours(3) most radioactivity was present in supernatant fraction of kidneys in bound form, and measurements at various time intervals after administration of B_{12}-Co^{60} (4) showed that kidneys progressively lose radioactivity. Intracellular distribution of radioactivity of administered B_{12}-Co^{60} in livers of rats(3,5,6) and of $cobalt^{60}$ in organs and intracellular organelles of mice(7) was studied. The relationships of amounts of Vit. B_{12} in fractions of kidneys and livers of normal and deficient animals at different time intervals after administration of vitamin has not been studied. We studied the intracellular distribution of B_{12}-Co^{60} and $cobalt^{60}$ in kidneys and livers of normal and B_{12} deficient rats at intervals after the last injection of radioactive compounds.

Methods. Rats of the St. Louis University colony were fed soybean meal diet of Hogan *et al.*(8) for production of deficiency of B_{12} and soybean meal diet with 90 μg Vit. B_{12}/kg of diet or Purina lab chow for control animals. Rats fed these diets for 8 to 10 weeks received 1 or 5 injections (1 μc each injection at 24 hour intervals) of $Co^{60}Cl_2$ or high specific activity B_{12}-Co^{60}.‡ At intervals after last injection, animals were sacrificed and samples of liver and kidney excised, weighed, homogenized in cold 0.88 M sucrose, and cellular components separated by differential centrifugation(9). Microsomal fractions were prepared by centrifuging 1 hour at 100,000 x g in a Spinco Model L centrifuge. Each sedimented fraction was washed twice with sucrose and the sediment suspended in 5 ml of sucrose for each gram-equivalent of original tissue used. There was good agreement between cts/min§ of minced tissue, homogenates and the sum of cts/min of the fractions. Radioactivity recovered in fractions was 88% to 102% of that in homogenates. Nitrogen was determined by micro-Kjeldahl procedure.

Results. Table I shows distribution of nitrogen in fractions of liver and kidney of normal and deficient animals. Normal group contains data on animals fed laboratory chow (Table II) and soybean meal diet supplemented with 90 μg of B_{12} (Table III). Separate treatment of data on nitrogen distribution showed no difference between groups so the data were combined. Nuclear and mitochondrial fractions of liver and kidney of deficient animals contain more nitrogen than these fractions of normal animals. Conversely, microsomal fraction of tissue of normal animals

* Aided by grant from Nat. Vitamin Fn.

† The authcrs wish to thank Agnes Kirkpatrick for valuable technical assistance in conducting nitrogen determinations.

‡ High specific activity B_{12}-Co^{60} (1121 μc/mg) was furnished through courtesy of Dr. Nathaniel S. Ritter, Merck Sharp & Dohme, Rahway, N. J.

§ Radioactivity was counted with a Tracerlab SC-57 low background well scintillation counter and an SC-18A scaler. cts/min = counts/min.

TABLE I. Distribution of Nitrogen in Fractions of Liver and Kidney Tissue. Values given are the mean ± standard error in terms of mg N/fraction equivalent to one gram of fresh tissue.

	No. rats	Homogenate	Nuclei	Mitochondria	Microsomes	Supernatant	Recovery total means
Normal liver*	14	37.07 ± 3.12	5.87 ± .61	6.03 ± .44	7.16 ± .52	17.54 ± 1.82	36.60
Deficient liver	22	39.87 ± 1.41	6.23 ± .36	7.38 ± .27	5.21 ± .28	19.10 ± .71	37.92
Normal kidney	14	31.62 ± 1.89	5.38 ± .46	5.26 ± .21	5.46 ± .47	14.98 ± .79	31.08
Deficient kidney	22	31.55 ± .91	6.06 ± .31	5.81 ± .09	4.17 ± .31	15.13 ± .52	31.17

* The term normal is applied to animals fed laboratory chow or soybean meal diet supplemented with 90 μg vit. B_{12}/kg and the term deficient is for those animals fed basal soybean meal diet.

contains most nitrogen. There was no marked difference in distribution of nitrogen (8 normal animals) or radioactivity (2 deficient animals injected with B_{12}-Co^{60}) in fractions obtained from 0.25 M sucrose by the modified procedure of Hogeboom(10) as compared with the data in the tables.

Eight animals fed laboratory chow received 5 injections of B_{12}-Co^{60} (5 μc) and 8 animals fed soybean diet (B_{12} deficient) were given B_{12}-Co^{60} (5 μc). Table II shows distribution of radioactivity in fractions of liver and kidney.

Kidneys concentrated radioactivity the most, but during 30 days, concentration of radioactivity declined. At 12 hours supernatant fraction of kidney tissue of both groups of animals contained the most radioactivity. However, at 30 days mitochondrial fraction of kidneys concentrated the most radioactivity. These data suggest a stronger affinity of mitochondria for radioactivity.

Homogenates of liver of control animals contained less than one-half as much radioactivity as livers of animals fed the B_{12} deficient diet. Mitochondria of liver under all conditions concentrated the most radioactivity. Furthermore, at 30 days radioactivity in mitochondria was 3 times as much as at 12 hours. This marked rise in liver mitochondria is a result of transfer of radioactivity to the liver from extrahepatic sources. Since whole kidney lost radioactivity during this period, it is one possible source of radioactivity.

These studies were repeated with a modified procedure in which 2 to 4 animals were used for each experiment. Animals were fed soybean meal diet or soybean meal diet with 90 μg of B_{12}/kg of diet. Animals consuming B_{12} deficient diet received 1 μc of B_{12}-C^{60} or Co^{60}

TABLE II. Intracellular Distribution of Radioactivity at Intervals of Time after Injection of B_{12}-Co^{60} (1 μc Given Daily for 5 Days). Values expressed as counts/min./mg N.

	Laboratory chow			Deficient*		
	12 hr 3 rats	12 day 2 rats	30 day 3 rats	12 hr 2 rats	12 day 2 rats	30 day 4 rats
Liver						
Homogenate	211	331	350	486	777	1,141
Nuclei	252	162	249	722	838	1,523
Mitochondria	364	607	1,294	816	1,752	2,402
Microsomes	104	146	66	194	275	336
Supernatant	194	371	382	543	675	782
Kidney						
Homogenate	20,616	13,363	9,208	30,840	23,967	8,600
Nuclei	15,310	11,499	3,665	16,637	18,081	4,355
Mitochondria	8,668	11,866	12,357	30,470	19,282	10,406
Microsomes	8,178	7,410	2,043	11,714	6,796	2,408
Supernatant	28,086	14,645	11,227	46,103	31,856	8,873

* Basal soybean meal diet deficient in vit. B_{12}.

TABLE III. Intracellular Distribution of Radioactivity at Intervals of Time after Injection of B_{12}-Co^{60} or $Co^{60}Cl_2$. Values are expressed as counts/min./mg N.

	Deficient;* 1 μc B_{12}-Co^{60} inj.			Deficient + 90 μg B_{12}/kg diet; 1 μc B_{12}-Co^{60} inj.			Deficient; 1 μc $Co^{60}Cl_2$ inj.		
	12 hr	12 day	30 day	12 hr	12 day	30 day	12 hr	12 day	30 day
	4	2	2	2	2	2	2	2	2
Liver									
Homogenate	161	401	309	68	117	70	290	159	31
Nuclei	150	341	288	60	62	63	291	155	98
Mitochondria	177	895	735	83	182	168	265	332	73
Microsomes	172	165	252	68	44	45	321	215	27
Supernatant	181	339	190	44	72	73	240	103	4
Kidney									
Homogenate	4,963	2,944	1,617	8,518	3,424	4,379	1,450	255	72
Nuclei	3,505	1,937	979	6,100	2,613	3,844	991	174	35
Mitochondria	3,115	5,715	3,174	11,667	4,066	5,906	2,240	528	129
Microsomes	3,992	1,314	501	1,769	909	1,262	487	306	70
Supernatant	6,470	2,738	1,393	9,062	5,021	4,637	1,279	150	48

* Basal soybean meal diet deficient in vit. B_{12}.

Cl_2 parenterally. Animals fed deficient diet supplemented with Vit. B_{12} received 1 μc of B_{12}-Co^{60}.

The results of this experiment are shown in Table III. The fate of radioactivity of $Co^{60}Cl_2$ is markedly different from that of B_{12}-Co^{60}. Kidney tissue retains much less radioactivity from $Co^{60}Cl_2$ than from B_{12}-Co^{60}. Furthermore, at 30 days there is little radioactivity from $Co^{60}Cl_2$ in kidney or liver tissue.

Discussion. Our results show that liver tissue of Vit. B_{12} deficient rats binds greater quantities of radioactivity of B_{12}-Co^{60} than liver of rats fed diets containing the vitamin. Our data at 12 hours and 12 days are in agreement with those of Barrows and Chow(3) taken at 48 hours, which show that supernatant fraction of kidney contains the greatest concentration of radioactivity from parenterally administered B_{12}-Co^{60}. However, over 30 days, radioactivity of supernatant fraction of kidney decreases more rapidly than that of the mitochondrial fraction.

The increase in concentration of radioactivity in rat liver with time after injection of 5 μc B_{12}-Co^{60} was concomitant with loss of radioactivity by kidney. Most vitamin thus obtained by liver was concentrated in the mitochondrial fraction. Therefore, these data are in agreement with those of Swenseid *et al.*(1), and possibly show interrelationship between kidney and liver. Since tissues of B_{12} deficient rats took up greater quantities of B_{12}-Co^{60}, use of isotopically labeled vitamin for tracing the fate of Vit. B_{12} may prove a useful tool for studying the metabolic role of the vitamin.

Rosenblum *et al.*(2) reported that most parenterally administered Vit. B_{12} was excreted unchanged in urine of rats within a short period of time. We compared the rate of disappearance of radioactivity of B_{12}-Co^{60} and $Co^{60}Cl_2$ from kidneys and livers of rats and found that inorganic cobalt is not retained to the same extent as is cobalt of Vit. B_{12}. The data suggest that radioactivity of B_{12}-Co^{60}, bound by mitochondria of liver and kidney, is deposited in these tissues in a form other than inorganic cobalt (presumably, Vit. B_{12}).

Summary. 1) In a study of intracellular distribution of radioactivity of B_{12}-Co^{60}, the mitochondrial fraction of livers of B_{12} deficient rats takes up more radioactivity than does this fraction of normal animals. This increases between 12 hours and 30 days. 2) At 12 hours the supernatant fraction of kidneys of normal and deficient animals is highest in radioactivity, however, at the end of 30 days, radioactivity in the mitochondrial fraction is greatest. 3) There is a transfer of radioactive B_{12} to liver from extrahepatic sources. 4) Radioactivity of $Co^{60}Cl_2$ is not retained by fractions of tissues as is that of B_{12}-Co^{60}.

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Received March 17, 1959. P.S.E.B.M., 1959, v102.

Distribution of Injected Heparin in Different Blood Fractions.* (25128)

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Although the effect of heparin in inhibiting coagulation of blood has been known for a number of years, almost nothing is known with respect to the mechanism by which this action is exerted. It is most probable that at least one step involves an interaction with one of the plasma proteins since the presence of a normal blood component is a necessary requirement in prevention of blood clotting by heparin. As an initial step experiments were, therefore, conducted to determine in which blood fraction the heparin resides subsequent to intravenous administration. When this is decided further studies could be made on the specific fraction involved. We employed heparin-S^{35} since this can be measured in minute amounts. The present report describes experiments on blood fractionation after intravenous injection of heparin and determination of radioactivity in the different fractions.

Methods. Heparin-S^{35} in the form of sodium salt was prepared as described previously(1). This material, having an anticoagulant activity of 152 U.S.P. units/mg, maintained a constant specific activity (8,010 c.p.m./mg) after repeated recrystallization. Other analytical data were as follows: glucosamine 23.22%; nitrogen 2.25%; sulfur 12.02%; sodium 7.88%; $[\alpha]_D^{25}$ + 51°. Three dogs weighing 8-9 kg were injected intravenously with solution of 30 mg of heparin-S^{35} (2.4×10^5 c.p.m.) in physiological saline. Blood samples drawn from animals 15 and 30 minutes after injection were employed in fractionations and subsequent radioactivity assay. First, blood was separated into plasma and cells. A second portion of blood was clotted and serum separated from clotted material was fractionated into components of different densities. After mixing a serum aliquot with aqueous sodium chloride to give a solution with density of 1.006, the mixture was centrifuged at 79,000 g for 20 hours at 4° (Spinco Ultracentrifuge, Model L). The top layer (density, less than 1.006) was removed for radioactivity analysis and the infranate was mixed with salt solution (D, 1.346 to give a final density of 1.063). The latter mixture was centrifuged at 79,000 g for 20 hours and the top layer (D, 1.006-1.063) taken off. The above procedure was repeated with the infranate using potassium bromide as the salt to give top layers with densities of 1.063 - 1.120 and 1.120 - 1.200. The different fractions were then combusted, converted to barium sulfate, and counted in gas flow counter. Another 10 ml of serum was mixed with 90 ml of 5% trichloroacetic acid. The precipitated protein was collected and washed with trichloroacetic acid. The washings were combined with the original supernate. In another experiment serum was mixed with 3 volumes of alcohol and the precipitated material separated from the supernate. Additional experiments were conducted in which plasma was submitted to chemical fractionation by Cohn

* Supported by grants from Atomic Energy Comm. and Am. Heart Assn.

TABLE I. Radioactivity of Blood Fractions after Intravenous Injection of Heparin-S^{35} in Blood Drawn 15 and 30 Min. after Injection.*

Fraction	Radioactivity, cpm/10 ml of blood 15 min.	30 min.
Whole blood	1688 ± 96	1034 ± 82
Cells	76 ± 9	22 ± 3
Plasma	1542 ±116	928 ± 15
Clot	42 ± 2	31 ± 2
Serum	1476 ± 96	841 ± 53
Dialyzed serum	1458 ± 89	830 ± 47
Alcohol precipitate	1452 ±104	810 ± 64
" filtrate	2 ± .6	3 ± 1
T C A precipitate	1402 ± 7	791 ± 19
" filtrate	21 ± 3	8 ± .9

* Based on avg of 3 experiments wherein each experiment was conducted in triplicate.

method IV(2) and the material separated into I + III, II, IV, VI, and albumin fractions. Finally, a serum aliquot was dialyzed against running water for 4 days at 4°, and the dialyzate was assayed for radioactivity. Radioactivity measurements were made on a gas flow counter and sufficient counts were taken to obtain an accuracy of ± 10%. Values obtained were converted to number of c.p.m. at infinite thinness.

Results. Figures obtained on radioactivity assay of the various fractions are a reflection of heparin concentration. This is demonstrated by the fact that radioactivity in blood is dialyzable (Table I). If desulfation and consequent removal of the label had occurred, the latter would be in the form of inorganic sulfate which can pass through the cellophane membrane.

Table I shows results obtained in terms of c.p.m. in various blood fractions. Almost no radioactivity (heparin) is taken up by blood cells and practically all material in plasma can be recovered in the serum fraction. Furthermore, both trichloroacetic acid and alcohol precipitation show that heparin is associated with proteins. Heparin itself, when dissolved in water cannot be precipitated with trichloroacetic acid.

Of special interest is the fact that no significant amount of radioactivity can be recovered in low-density lipoproteins upon centrifugation in media of different density (Table II). This excludes the possibility of association of heparin with chylomicrons and the major portions of α-lipoproteins. The reported effect of heparin on chylomicrons(3) and lipoproteins(4) apparently does not involve any chemical combinations with these substances and must operate by some indirect action. Most probably this involves interaction of heparin with lipoprotein lipase(5) which in turn elicits the lipolytic effect.

Chemical fractionation of plasma and radioactivity assay of different fractions indicates that the major portion of heparin is found in fraction I + III and II (Table III). It is noteworthy that these fractions contain some of the clotting proteins, *i.e.*, fibrinogen and prothrombin, in addition to γ-globulin and β_1-lipoproteins. Whether heparin combines with one or several of the proteins of these fractions cannot be decided from these experiments and our present studies are concerned with this question. The finding that the albumin fraction contained only traces of radioactivity was somewhat surprising since interaction of heparin with albumin has been demonstrated by electrophoretic studies of Chargaff, *et al.*(6). However, it should be noted that experiments in which such binding was demonstrated were conducted with comparatively high concentrations of heparin. In our study and in clinical administration, actual blood heparin concentration is minute. It

TABLE II. Distribution of Radioactivity in Serum Fractions of Different Density. 50 ml of serum from blood drawn 30 min. after injection were employed. (Avg of 3 exp.)

Fraction	cpm in total
Serum	5166 ±320
D, 1.006	13 ± 1
" -1.063	18 ± .6
1.063-1.120	0
1.120-1.200	14 ± 2
1.200	5062 ± 29

TABLE III. Radioactivity in Plasma Fractions after Chemical Fractionation. 10 ml of plasma obtained from blood 30 min. after injection.

Fraction	Radioactivity, cpm
Original plasma (10 ml)	928 ± 15
Fraction I + III	606 ± 39
II	93 ± 10
IV	14 ± 1
VI	16 ± 3
Albumin	8 ± 1

is quite possible, therefore, that interaction of heparin with albumin does not occur under these conditions.

Heparin is a highly charged molecule and it is reasonable to expect that the mechanism for its actions involves some protein interaction through these charges. Although studies have demonstrated interaction of heparin with individual proteins, comparatively little is known of how it reacts when administered in the bloodstream. The present study narrows the possibilities of reaction to the proteins of certain specific fractions.

Summary. 1. After intravenous injection of heparin-S^{35}, radioactivity is concentrated in the serum fraction. Only minute amounts of radioactivity are found in low density lipoproteins. Chemical fractionation shows that the preponderant portion of radioactivity is in Cohn fractions I + III, lesser amounts in fraction II and only minute quantities in other fractions. 2. Heparin is not desulfated in the bloodstream.

The authors gratefully acknowledge assistance of Messrs. Roger Casciano and Hosein Payinda.

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Received March 23, 1959. P.S.E.B.M., 1959, v102.

Development of a Canine Globulin Concentrate. (25129)

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Antiserum prepared from blood of dogs hyperimmunized against distemper has been used to aid in controlling this disease since 1925. More recently, antigens of *Leptospira canicola* and infectious canine hepatitis virus have been added to the hyperimmunizing antigens to make available a single antiserum containing specific antibodies against each of these major diseases. In some cases antigens of common bacterial secondary invaders also are used. Multivalent antiserums prepared in this manner have proved valuable for conferring passive immunity for immediate protection against the agents used for hyperimmunization, and as a part of therapeutic programs after exposure. Presently available antiserums have 2 outstanding disadvantages: (1) antibody content is not precisely standardized, and (2) they contain large amounts of immunologically inert proteins. A degree of standardization may be attained by using large numbers of donor dogs and bleeding repeatedly so that turnover in donor population is gradual and influences antibody content only slightly. Advances in immunochemistry have made purification and concentration of antibody proteins possible, as well as more precise methods of assay. Such improvements were instrumental in developing gamma globulin in human medicine. They also have provided means for developing similar preparations in veterinary medicine. This report is concerned with development of a purified canine globulin concentrate, derived from blood of hyperimmunized dogs, and presents results obtained with this concentrate.

Methods and materials. Starting material used for preparation of globulin concentrates was plasma obtained from pooled blood of hyperimmunized adult dogs. The hyperimmunizing process involved weekly injections of canine distemper virus, infectious canine he-

patitis virus, *Leptospira canicola,** and a mixed bacterin prepared from cultures of *Salmonella typhimurium, Brucella bronchiseptica,* and *Streptococcus* sp. Each lot was prepared from plasma of at least 100 donor dogs. Blood was collected into sodium citrate solution, and the plasma separated by centrifugation. Plasma was defibrinated by first heating at 54°C for one hour and cooling rapidly. Fibrin was removed from the serum by passing through coarse screens and centrifuging. To the clarified serum, at pH 6.8, methanol was added to final concentration of 25% while temperature of the mixture was brought to −6°C. A precipitate which formed was separated from the supernate by Sharples centrifugation. This precipitate contained most of the beta and gamma globulins, while albumins and most other serum proteins remained in the supernatant fluid. The precipitate was freed of alcohol by drying from the frozen state. After desiccation, the dried powder was restored to one-fifth the volume of the original serum and filtered to remove bacteria. Animal protection tests were conducted in littermate puppies, except in a few instances when hamsters were used for studies with *Leptospira canicola.* Litters of puppies were purchased from farms when 10 to 20 weeks of age, and immediately removed to isolation quarters. Isolation units were constructed of concrete and cement plaster so that they could be thoroughly disinfected and cleaned with hot water, and were supplied with filtered air under continuous positive pressure. Titrations comparing whole serum pools (parent serums) and their globulin concentrates were carried out in single litters of puppies. Parent serums and purified globulin concentrates were administered in measured volumes/lb of body weight. Replicate tests with various lots were made in several litters of dogs to determine respective minimum protective dosages. Serum or its purified globulin concentrate was given subcutaneously 24 to 48 hours prior to challenge with the disease agent under study. Dogs challenged with canine distemper virus were exposed both by parenteral inoculation of virulent virus, and by contact with distemper-infected dogs. For this challenging procedure dogs were removed from isolation pens and taken to kennels used for production of canine distemper virus. Here they were injected subcutaneously with 1 to 2 cc of a suspension of spleen tissue containing virulent virus, after which they were placed in pens for 6 to 24 hours with dogs previously infected with canine distemper. They were then returned to isolation units, observed for 21 days, and daily temperature records kept. Whenever clinical observations were inconclusive with respect to canine distemper infection, a complete postmortem and histopathologic examination of the dog was made when the observation period terminated. Challenge with infectious canine hepatitis virus was by intravenous injection of tissue culture fluids with high concentrations of virulent infectious canine hepatitis virus. The challenge virus was isolated from a dog in the terminal stage, and carried in tissue culture for only a few passages. Dogs challenged with infectious canine hepatitis virus were observed 14 days, during which daily white blood cell counts and temperature recordings were made. Challenge with *Leptospira canicola* was carried out by intraperitoneal inoculation of strains highly virulent for the test species (dog or hamster). Test animals were observed 14 days after challenge. Susceptible dogs showed typical symptoms of leptospirosis and usually became severely icteric, while susceptible hamsters usually died within 10 days. In testing antiserums and their concentrates for protective potency against exposure to the various diseases, chief emphasis was placed on protection against canine distemper virus. This course was followed for 2 reasons: (1) canine distemper is the most widespread and difficult to control of the major specific infections of dogs, and (2) smaller amounts of serum or concentrate protect against infectious canine hepatitis or leptospirosis than against canine distemper. The various lots of parent serums and concentrates also were tested serologically for antibody content. Neutralization titers against canine distemper virus were determined by procedures generally following those described by Baker, Gorham and Leader(1).

* Cultures stored in frozen state and thawed just before use.

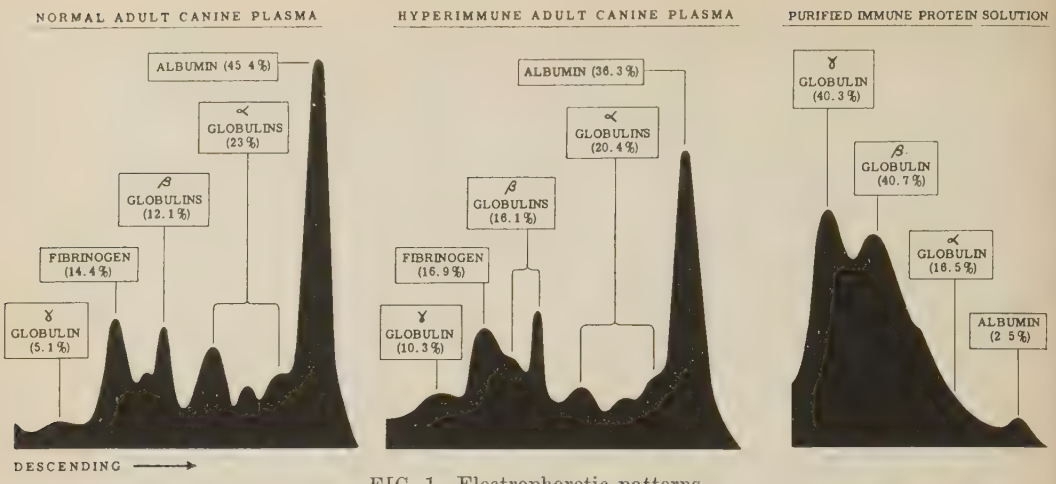

FIG. 1. Electrophoretic patterns.

Dilutions of serum or concentrate were mixed with uniform amounts of an egg-adapted test strain of canine distemper virus, incubated, and the mixtures inoculated on the chorioallantoic membranes of developing chick embryos. Presence or absence of typical lesions after incubation indicated the neutralizing capacities of various dilutions of serum. Infectious canine hepatitis virus neutralization tests were made in trypsinized dog kidney tissue culture systems as described by Cabasso and associates(2) and Fieldsteel and Emery (3). Leptospira antibodies were measured by the agglutination-lysis technic described by Coffin and Stubbs(4). Paired blood samples taken from dogs before and after injection of serum or globulin concentrate also were tested to determine how much passive antibody appeared in the blood of the recipient.

Results. Early in the study, pure gamma globulin of hyperimmune canine serum was found to contain only about 50% of the total antibody, and all of the antibody was recovered only if both gamma and beta globulin fractions were included. Electrophoretic analysis showed that concentrate obtained by the method described is a solution of proteins, 80% or more of which consist of gamma and beta globulins. Fig. 1 shows typical electrophoretic patterns of normal canine serum, hyperimmune canine serum, and globulin concentrate of hyperimmune canine serum.

Comparative studies showed that amounts of beta and gamma globulins present in parent hyperimmune serum pools and their purified concentrates were quite consistent, and antibody titers of either material tended to follow total concentrations of beta and gamma globulins (Table I).

The results of challengng 132 dogs in 20

TABLE I. Antibody Titers and Globulin Contents of Hyperimmune Canine Serum and Globulin Concentrates.

Lot	Product	Globulin content, mg/ml	Canine distemper titer*	Infectious canine hepatitis titer†	*L. canicola* titer‡
1	Globulin conc.	119	46,000	15×10^4	4096
	Hyperimmune canine serum	29	11,000	$5 \times$ "	1024
2	Globulin conc.	104	31,000	$15 \times$ "	4096
	Hyperimmune canine serum	21	10,000	$5 \times$ "	2048
3	Globulin conc.	102.5	36,000	15×10^5	4096
	Hyperimmune canine serum	21.5	6,000	15×10^4	512

* ND_{50}/1 cc determined by egg serum-virus neutralization test at level of 112 EID_{50} of virus /egg dose.

† ND_{50}/1 cc determined by tissue culture serum-virus neutralization test at level of 1000 $TCID_{50}$ of virus/tissue culture dose.

‡ Reciprocal of highest dilution giving a 2+ reading as determined by agglutination lysis test.

TABLE II. Determination of Protective Dosage (50%) and Globulin Content of Hyperimmune Serum and Concentrate.

	Canine distemper		Infectious canine hepatitis		*Leptospira canicola*	
Dosage	Results of challenge	50% protective dose mg globulin	Results of challenge	50% protective dose mg globulin	Results of challenge	50% protective dose mg globulin
Globulin concentrate		4.3		.96		3.8
1.0	7/7					
.5	8/8					
.2	6/7		2/2			
.1	7/7		"		2/2	
.05	3/4		1/1		1/1	
.025	1/4		"		0/2	
.0125			2/2			
.00625			0/1			
Hyperimmune serum		3.8		1		<2.9
1.0	4/4		2/2			
.5	11/11				2/2	
.25	4/4		1/1		1/1	
.125	1/4		"		2/2	
.0625			2/2			
.03125			0/1			
Controls	2/28		0/10		1/4	

Denominator = No. of dogs tested; numerator = No. of dogs remained well.

litters are summarized in Table II. Many other tests are not included because the littermate control puppies either were immune, or failed to react typically to challenge.

While 50% protective dosage volumes of concentrates and parent serums are very different (Table II), each contains approximately the same amount of gamma and beta globulins/dose, indicating complete recovery of antibody with the beta and gamma globulin fraction.

Protective endpoints of the 3 lots of concentrates and parent serums against canine distemper virus compared rather closely in terms of mg of globulin (Table III). While the 50% endpoint results shown in Table III indicate some difference in antibody recovery between Lots 2 and 3, the number of dogs involved in testing individual lots may be too small to consider the difference reliable. However, there were minor variations in the defibrination process, and clarification and filtration of Lot 2, while Lot 3 was prepared exactly as outlined previously.

In addition to tests in dogs for determining relative potency of antiserum and concentrate against *Leptospira canicola,* concentrate Lots 1 and 2 and their parent serums also were

TABLE III. Comparative Protective Doses for Canine Distemper and Their Globulin Content.

	Lot #1		Lot #2		Lot #3	
Dosage (cc/lb)	Results of challenge	50% protective dose mg globulin	Results of challenge	50% protective dose mg globulin	Results of challenge	50% protective dose mg globulin
Globulin concentrate		<3.0		5.2		3.6
.2	2/2		4/5			
.1	1/1		5/5		1/1	
.05	"		1/2		"	
.025	"		0/2		0/1	
Hyperimmune serum		5.2		2.7		3.8
1.0			4/4		1/1	
.5	1/1		9/9		"	
.25	"		2/2		0/1	
.125	0/1		1/2			
Controls	1/7		0/18		1/3	

Denominator = No. of dogs tested; numerator = No. of dogs remained well.

TABLE IV. Protection Tests with Hyperimmune Canine Serum and Globulin Concentrate in Hamsters. Challenging agent was *Leptospira canicola.*

	Lot #1		Lot #2	
Dosage = 0.3 cc of dilution indicated	Results of challenge	50% protective dilution	Results of challenge	50% protective dilution
Globulin concentrate		1:64		1:45
1:4	4/4		4/4	
1:8	"		3/4	
1:16	"		4/4	
1:32	3/4		3/3	
1:64	"		1/4	
Hyperimmune serum		1:12		1:7
1:4	3/4		3/4	
1:8	4/4		2/4	
1:16	1/4		0/4	
Controls	0/3		0/3	

Denominator = No. of hamsters; numerator = No. protected.

tested in hamsters. These tests indicated a somewhat greater difference in hamsters between protective doses of parent serums and their concentrates (Table IV).

Antibody concentrations demonstrable in serums of littermate puppies which had received identical doses of antibody/lb. body weight sometimes varied considerably (Table V). However, at higher dosages, puppies were resistant to challenge with virulent canine distemper virus whether their serum titer after administration of antibody was high or low. At lower dosages, some puppies with no demonstrable serum antibodies resisted challenge with virulent distemper virus, whereas some puppies with low titers developed distemper.

Discussion. That the active fraction can be separated from hyperimmune canine serum in highly purified form without significant loss or destruction of antibody is of considerable clinical importance. Comparatively, purified globulin concentrates, like parent hyperimmune serums, are effective in smaller dosages for protection against *Leptospira canicola* or infectious canine hepatitis than against canine distemper.

Purification of the antibody carrying fraction of serum opens the way for standardization of antibody content by standardizing the amount of globulin contained in unit volumes of purified concentrate. In this way, variations in concentration of antibody in serum pools can be nullified, whereas concentration based on volume alone would cause each lot of concentrate to reflect variations between lots of parent antiserums. Knowing the antibody globulin content of the concentrate, and minimum average dose for short-term passive protection, it would be possible to administer antibody in multiples of the minimum dose for special purposes in more precise dosage than has heretofore been possible.

That resistance to distemper correlated well with amount of antibody administered/lb. body weight, but less precisely with the level of antibody demonstrated in the blood, indicates that passively acquired antibody may be active in tissues and body fluids other than blood.

In addition to the demonstrated immunizing activity of concentrated globulins in these experiments, field trials may well be expected to demonstrate a wider usefulness. In commercial lots, bleedings from 1000 or more dogs, originating over a wide area, are incorporated in each pool of plasma. It is assumed that, in addition to antibodies resulting from specific hyperimmunizations, the purified globulin concentrates may contain other antibodies normally present in adult canine populations exposed to various infectious agents. While they would be present also in parent serums, their concentration before processing would be so low that protective doses would be unreasonably large for practical use. As with human gamma globulin(5), the concentrated canine globulin might make significant amounts of such common antibodies available in practical dosage. If the concentrate could

TABLE V. Canine Distemper Antibody Titers after Various Dosages of Globulin Concentrate or Hyperimmune Serum, and Results of Challenge with Virulent Distemper Virus.

Litter	cc/lb	CD antibody titers* Preinoc.		24 hr postinoc.	Results of challenge
	Globulin conc.				
I	1	Neg.†	1:10	260	Remained well
"	"	"	"	220	*Idem*
H	"	"	"	310	"
"	"	"	"	220	"
I	"	"	"	220	"
"	"	"	"	150	"
H	"	"	"	220	"
"	"	"	"	180	"
I	.2	"	"	<100‡	"
"	"	"	"	"	"
H	"	"	"	Neg.	1:10 "
"	"	"	"	"	" "
L	.1	"	"	"	1:5 "
M	"	"	"	"	" "
L	.05	"	"	"	" "
M	"	"	"	<50‡	"
L	.025	"	"	"	1:5 CD
M	"	"	"	"	" Remained well
	Hyperimmune serum				
L	.5	Neg.	1:10	Neg.	1:5 *Idem*
M	"	"	"	50	"
L	.25	"	"	"	1:5 "
M	"	"	"	"	" "
L	.125	"	"	<50‡	CD
M	"	"	"	"	1:5 "

Susceptibility to distemper was demonstrated for all litters by characteristic response to challenge of untreated, serologically negative controls.

* ND_{50}/1 cc: Titers determined against following amounts of virus: litters I and H, approximately 100 EID_{50}, and litters L and M, approximately 130 EID_{50}.

† Neg. = no antibody demonstrated at lowest dilution as indicated.

‡ <100 or <50 = some antibody demonstrated at lowest dilution (1:10 or 1:5).

be used in control of other common infections characterized by such symptoms as cough, diarrhea or skin eruptions, it probably would have to be used in larger dosages than those required for control of diseases against which the donor dogs were specifically hyperimmunized. Again, as with human gamma globulin, such possible benefits of purification and concentration of canine globulins can be revealed only after sufficient clinical evaluation.

Summary. A method is described for purifying and concentrating antibody globulins from serum of dogs hyperimmunized against canine distemper, infectious canine hepatitis and *Leptospira canicola*. Our data show that concentrated canine globulins contain all the protective antibody of serum. The method should make possible standardization of antibody content. Possible additional usage of concentrated canine globulins, analogous to clinical usage of human gamma globulin, has been suggested.

The authors acknowledge the invaluable technical assistance of W. H. Dazey, F. B. Gauker, and J. W. Newberne.

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Received April 17, 1959. P.S.E.B.M., 1959, v102.

The Response of Mouse Neurons to Mouse Sarcomas 37 and 180.* (25130)

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Mouse sarcomas 37 and 180 stimulate differentiation of sympathetic and sensory ganglia in young chick embryos(1,2,3). In these ganglia the growth rate of individual cells is accelerated and the final cell population is greater than that in controls; the motor system is unaffected. This response is mediated by a protein produced by tumor cells(4). An analogous response occurs under *in vitro* conditions, the pattern of fiber outgrowth from chick ganglia being characteristicalliy altered in presence of explants(5) or extracts(4) of the tumors. More recently proteins with identical effects but of much higher potency have been found in snake venoms(6,7) and mouse salivary glands(8,9). The reaction of chick neurons to this substance seemed interesting enough to warrant exposing the developing neurons of other vertebrates to these tumors. In our experiments the responses of the mouse nervous system during fetal and early postnatal stages were studied.

Materials and methods. Spinal ganglia from BUB mice ranging in age from 11th prenatal to 10th postnatal day were grown in plasma clot cultures. In the experimental series pieces of sarcoma 37 or 180 were placed close to the ganglia. In some experiments sympathetic ganglia or pieces of spinal cord were tested, but sensory ganglia were most often used. Parallel studies of chick material served as a standard of comparison. Observations and measurements were made on cultures at intervals after about 12 hours incubation. Fixed material was studied after staining by the silver-reduction method of Cajal-DeCastro or Holmes.

Results. In control cultures the pattern of outgrowth from mouse ganglia is very similar to that from chick ganglia. Neurites first appear after about 12 hours incubation and are accompanied by a migrating population of spindle cells originating from the neural crest and abundantly present in all ganglia. The chief characteristic of fiber outgrowth in these cultures is the tortuous course taken by individual neurites in their generally radial route from the ganglion.

Several differences in the character of outgrowth were noted among ganglia from different age groups. Ganglia from 11- to 13-day fetuses are in the very early stages of differentiation and produce few neurites after explantation. Ganglia isolated between 14th day and term produce an abundance of fibers; ganglia from postnatal animals tend to be retarded (up to 24 hours) in initiation of outgrowth and in general produce fewer neurites (Figs. 1, 2).

In experimental cultures, neurites begin to appear after 9 hours incubation, *i.e.*, at least 3 hours earlier than in controls. The number of neurites far exceeds that in control cultures, and the path of each neurite is a relatively straight radial one. Usually the closely spaced fibers advance in unison, their tips appearing to lie on an invisible boundary of circular or elliptical shape. This phenomenon has been described in chick ganglia(5), and has been termed a "halo" effect. In ganglia of most age groups, migration of spindle cells is completely suppressed for the first 24 hours of incubation. This suppression is gradually overcome, as is the strict regulation of neurite advance. As was true in the chick, sympathetic ganglia respond similarly while neurons of spinal cord are unaffected.

The most interesting age group among experimental cultures is that of the ganglia isolated between 11th and 13th prenatal day. The modestly differentiated cells of these ganglia respond vigorously to the tumor substance and produce large numbers of fibers, whereas in control cultures they produce almost none. On the other hand, the spindle cell element of

* Part of thesis presented at Brown University for Ph.D. degree. This work was done during tenure of U.S.P.H.S. fellowship and supported in part by Institutional Grant from Am. Cancer Soc.

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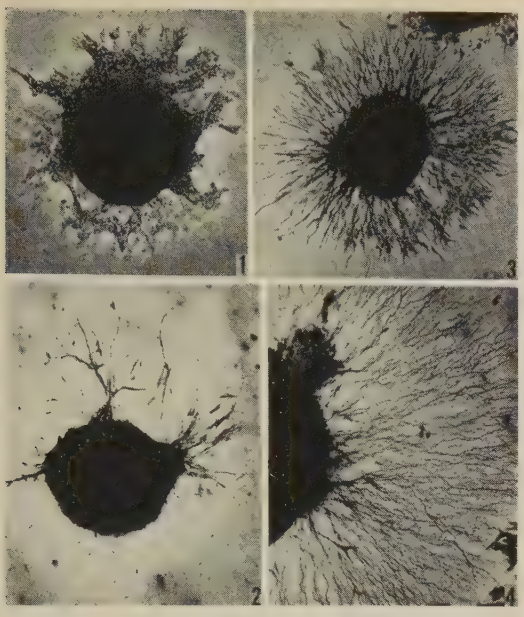

FIG. 1-4. Mouse spinal ganglia in culture. Holmes' silver method. ×35.

FIG. 1. From 11-day fetus; incubated 36 hr; a few neurites at upper right.

FIG. 2. From newborn; incubated 36 hr; few neurites and spindle cells.

FIG. 3. From 12-day fetus; incubated 20 hr with tumor (upper right); many neurites and spindle cells.

FIG. 4. From newborn; incubated 36 hr with tumor (lower right); many neurites; spindle cells beginning to emerge.

these ganglia has not yet acquired sensitivity to the tumor substance; these cells fail to show any suppression (Fig. 3). Ganglia isolated at later stages show responses identical to those previously reported for the chick. Ability to respond under *in vitro* conditions extends at least through the first postnatal week (Fig. 4); the generally slower growth of ganglia isolated from older animals makes their response difficult to test under conditions of these experiments.

Additional information on the nature of the response is provided by measurements of fiber lengths made on about 200 living and on many more fixed cultures. Calculation of average rate of fiber elongation during the first 36 hours incubation showed no significant difference between control (7-10 μ/hour) and experimental (7-12 μ/hour) values. Measurements made on cultures in which the distance between ganglion and tumor was sufficient to produce initial differential effects at near and remote poles of the ganglion showed no consistent difference in fiber lengths in the 2 regions at 36 hours.

Attempts to alter development of the nervous system *in vivo* included implantation of tumors subcutaneously in mice during early pregnancy, injection of tumor brei into 16-day fetuses, and subcutaneous implantation in newborn animals, which in some instances had partially amputated forelimbs. The first 2 of these experiments suffered from the fact that sarcomas 37 and 180 do not thrive in pregnant animals. This had been reported for 180(10), and was true of 37 in this study. It is, therefore, unlikely that any animal was actually exposed prenatally to the tumor substance. Tumors implanted in postnatal animals grew rapidly to enormous size without effect upon the intact or injured nervous system in the 2 weeks that the animals survived. The detailed similarity of *in vitro* responses of chick and mouse neurons seems to indicate the possibility of an *in vivo* response from the mouse if proper conditions be found.

Discussion. In evaluating responses of mouse ganglia to the tumor substance, age differences in growth potential must be taken into account. It is impossible to determine whether the few neurites produced *in vitro* by ganglia isolated between the 11th and 13th prenatal day are regenerated from cells which had already produced fibers, or represent newly differentiating cells. In either case it is evident that very few cells isolated at these early stages are able to establish neurites in control cultures. Progressive retardation of initial outgrowth in ganglia from older animals may result from increased trauma to cells which have achieved more elaborate peripheral relationships; or it may simply be due to the phenomenon well known in tissue culture of the slower initiation of activity in older tissues.

Regardless of the interpretation given to the above phenomena, it has been amply demonstrated that the tumor substance has a stimulatory effect upon explanted mouse ganglia from very early stages throughout most if not all of their developmental course. The altered growth pattern described here is exactly simi-

lar, but for minor differences in timing, to that already reported for the chick. There is no explanation for the changed character of neurite growth under these conditions. There can be no doubt that the tumor substance is effective in eliciting fiber formation from cells which would be unable to produce neurites independently; similarly it can cause this process to occur precociously in cells which are themselves able to establish neurites. The substance is, moreover, responsible for the comparatively straight course followed by neurites in experimental cultures. The rate of axon elongation, however, is not increased.

Selectivity of cell type responsive to this substance is one of its more intriguing aspects. In both chick and mouse the motor system is totally refractory, the sensory and sympathetic systems highly responsive. In the chick a further subdivision is possible since the developing sensory ganglia apparently contain 2 morphological and functional groups of neurons. The small, mediodorsal cells are stimulated by the tumor substance; the large, ventrolateral cells are not detectably affected. Since in mouse ganglia corresponding groups of cells are not distinguishable, the comparison cannot be extended to this level.

Further evidence of specificity of responsiveness to the tumor substance is gained by considering the behavior of the spindle cells. The characteristic reaction of these cells in cultures containing pieces of tumor is a failure to migrate for at least 24 hours. After the period of suppression these cells move out from the ganglion in their usual fashion and number. The only exception to this behavior yet reported is the lack of suppression in cultures of ganglia from very young mouse fetuses. Thus, at a stage when the neural elements of sensory ganglia are already responsive to the tumor substance, spindle cell elements are apparently still undifferentiated in this respect. That the inhibition of movement is not a general response extending to fibroblasts is indicated by lack of any effect on chick heart fibroblasts(5) and on mesenchymal cells from mouse limb buds. Sheath cells migrating from segments of pre-degenerated peripheral nerves explanted from young mice are similarly unaffected. Thus, it would seem from limited experiments that suppression by the tumor substance is restricted to a specific class of cells during a particular period of development. That suppression is overcome after 24 hours is probably due to a decrease in amount of tumor substance present; there is a concurrent lessening of effect upon neurites. It is possible that tumor cells *in vitro* lose their ability to produce effective amounts of the critical substance.

Summary. 1) Dramatic responses to a tumor-produced substance occur in cells from mouse as well as chick ganglia. The knowledge that these responses are not unique to the chick adds to the interest which this phenomenon already had for the neuro-embryologist. 2) After conclusion of these experiments, the responsiveness of mouse and rat ganglia to the much more potent snake venom and salivary gland factors was reported(8,9).

The author wishes to thank Dr. Mac V. Edds, Jr. under whose guidance this work was done. The kind encouragement of Dr. Viktor Hamburger is much appreciated.

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Received April 17, 1959. P.S.E.B.M., 1959, v102.

Vascular Effects of Nylidrine Hydrochloride During Exercise. (25131)

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Various agents are capable of influencing regional blood flow. The vascular system apparently does not react as an entity to these agents; responses produced are neither uniform nor unidirectional in different vascular beds(1,2). The classical example of a drug, exhibiting both vasodilating and vasoconstricting activity at the same time is adrenaline, which dilates blood vessels of skeletal muscle and simultaneously constricts cutaneous vessels(3,4). There are situations in which vasodilation in muscle without vasoconstriction in skin is the desired effect. The first drug, fulfilling above criteria, was nylidrine hydrochloride (phenyl 2 butylnorsuprifen hydrochloride) known commercially as Arlidin,* chemically related to adrenaline and ephedrine. Several workers(5,6,7,8,9) found nylidrine caused dilation of blood vessels in the resting muscle without concomitant effects on skin. Exercise is the specific physiologic stimulus for producing vasodilation in skeletal muscle. Blood flow response is accompanied by little or no change in surface temperature. Consequently it seemed interesting to test the influence of nylidrine upon blood flow response to exercise.

Methods and material. Resting blood flow to foot and leg was measured by a large limb venous occlusion plethysmograph in 10 subjects in 20 experiments. Of these 10 subjects, 3 were without clinical evidence of cardiovascular disease; 7 patients had demonstrable non-gangrenous occlusive arterial disease. Surface temperature was recorded intermittently with a 6 lead Speedomax. All measurements were performed in constant temperature room at 20°C and 55% humidity with subject under basal conditions. After measurements of resting flow had been determined, the subject exercised for 5 minutes by pressing down 60 times/minute on foot board adjusted within the plethysmograph casement(10). Blood flow recordings were made immediately after exercise and every 2 minutes thereafter. When blood flow returned to pre-exercise base line levels, nylidrine (7 mg) was administered intravenously. After 15 minutes the exercise was repeated. Blood flow was measured every 2 minutes for 1 hour thereafter. All experiments were duplicated; in a number of experiments 2 exercise tests were done in succession, without drug administration. In a few experiments, tolazoline hydrochloride (50 mg) and azapetine (10 mg) were used for comparison.

Results. Resting blood flows ranged from 8.3 to 14.6 ml/100 ml tissue/min. in 3 normal subjects and from 4.7 to 8.1 ml/100 ml tissue/min. in 4 patients with occlusive arterial disease; in one sympathectomized patient blood flow was 11.4; in 2 who had been on oral Arlidin medication for several months prior to test, basal flows were 13.1 and 15.6 respectively. In response to first exercise test peripheral blood flow increased in all 10 subjects average 65.5%. Surface temperature rose in 3 subjects, average 4.6°C and did not show any essential change in 7 subjects (less than 2°C). When the exercise test was repeated after intravenous nylidrine (7 mg), 9 of 10 subjects showed average blood flow increase of 112.6%, exceeding that of initial exercise test by 47.5%. Surface temperature showed a small increase in 3, averaging 2.8°C; it decreased in 2 subjects, there was no significant change in surface temperature in 5. One subject with chronic sequelae of frost bite failed to show an increase in exercise response after nylidrine HCl.

Table I lists individual responses in all 10 subjects.

Fig. 1 shows a typical experiment. Control studies using repeated standardized tests without administration of nylidrine did not show any further increase in measured blood flow,

* Supplied through courtesy of U. S. Vitamin Corp. (Arlington-Funk Labs. Division) N.Y.C.

TABLE I. Blood Flow Response in Lower Extremities to Standard Exercise before and after I.V. Nylidrine HCl 7 mg to 10 Subjects.

Age	ml/100 cc tissue/min. Basal flow	After exercise	%	Surface temp. change, °C	ml/100 cc tissue/min. after exercise and nylidrine	%	Surface temp. change, °C	Remarks
68	8.3	12.9	55	−1	14.9	80	−1.5	Normal
50	12.1	19.0	57	−1	24.0	100	0	"
38	14.6	19.9	36	+4	22.5	54	−2	"
76	8.1	10.8	33	+3	13.0	60	+2.5	Occlusive arterial disease (compensated)
38	11.4	19.4	70	+1	23.0	100	+1.5	Sympathectomized occlusive arterial disease
61	6.7	12.9	92	0	18.7	180	0	Occlusive arterial disease (compensated)
50	6.4	13.5	110	0	14.7	130	0	*Idem*
36	4.7	11.0	130	+1.5	8.7	85	−2	Frost-bite
62	13.1	17.6	34	+1.5	21.8	66	+3	Occlusive arterial disease (4 mo on oral Arlidin, prior to exp.)
64	15.6	31.7	103	+7	53.5	250	+3	*Idem* (9 mo on oral Arlidin, prior to exp.)

above level obtained on initial exercise. When tolazoline and azapetin were used for comparison, only negligible additional increases in rate of blood flow in response to exercise were obtained, while surface temperatures showed a significant rise.

Comment. Additional increase in blood flow of 47.5% in response to exercise after nylidrine was never accompanied by additional increase in surface temperature; on the contrary, in general, temperature readings were lower compared to response to exercise without nylidrine administration. In subject E. F. *e.g.* surface temperature increased by 4°C on initial exercise, but decreased by 2°C on exercise after nylidrine. It can be reasonably assumed that additional increment in blood flow after nylidrine goes mainly to the muscle. It seems unlikely that shunting of blood from skin bed to muscle bed occurs since surface temperature usually rises slightly in response to exercise, though less after than before nylidrine administration. It has been shown by Barcroft *et al.*(11) that basal flow in resting muscle is regulated by vasomotor centers, but that this neurogenic regulation is not responsible for circulatory changes during muscle activity. Existence of 2 separate sets of minute vessels in skeletal muscle of man, only one of which is in direct connection with vessel branches supplying the skin, has been demonstrated by Saunders *et al.*(12). It seems most likely then, that circulatory changes in resting muscle occur simultaneously with circulatory changes in the skin in response to stimuli(13,14). In contrast, circulatory changes during muscular activity are chiefly dependent on the action of metabolites produced during such activity(15) and the skin bed hardly participates in these responses (10). Wiemers(16) showed in cats and dogs,

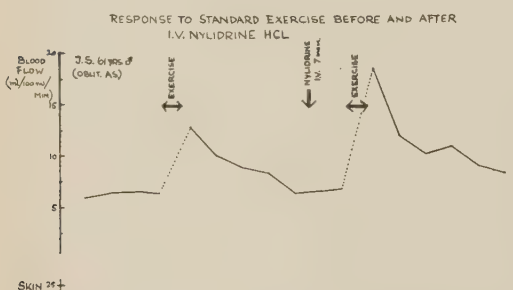

FIG. 1.

that depot blood was released from spleen and liver in response to nylidrine, without altering essentially the blood flow of remaining splanchnic bed. It is conceivable that most of this additional blood went to the periphery. A similar mechanism may perhaps be involved in our experiments.

There were no significant differences between directional and magnitudinal responses of normal subjects compared with patients with non-gangrenous occlusive arterial disease.

Summary and conclusions. Blood flow response to exercise was tested plethysmographically in 3 healthy subjects and 7 patients with non-gangrenous occlusive arterial disease of lower extremities, before and after intravenous administration of nylidrine hydrochloride, an adrenaline analog. Response in blood flow was significantly augmented after administratino of drug in all but one subject, who had severe bilateral frost bite. Since blood flow response to exercise takes place mostly in working skeletal muscles, it is concluded that the primary site of nylidrine action is in the vasculature of skeletal muscle. Nylidrine apparently enhances capacity of muscle vessels to respond to exercise.

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Received April 20, 1959. P.S.E.B.M., 1959, v102.

Fresh, Disintegrated Platelets in Radiation Thrombocytopenia: Correction of Prothrombin Consumption without Correction of Bleeding.* (25132)

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Platelets have 2 major functions in the hemostatic mechanism. The first is a mechanical action of intact platelets, the platelet plug(1) being the visible result of this effect, and the second is a complex biochemical role in coagulation. Many factors are involved, but the prothrombin consumption test may be taken to reflect the more important of these factors. It is essential to separate these 2 effects both from a theoretical and a practical point of view. Theoretically it should be known whether a normal clotting system, including platelet factors is able to maintain hemostasis in absence of mechanical effects of platelets. Practically, it should be known whether thrombocytopenic patients may benefit from platelet substitutes. Much and conflicting work is going on in this field(2,3,4), but it is not known whether normalization of the clotting system by disintegrated platelets will stop bleeding in thrombocytopenia. The value of the irradiated thrombopenic dog or rat in evaluating factors concerned with he-

* Supported by U. S. Atomic Energy Comm.

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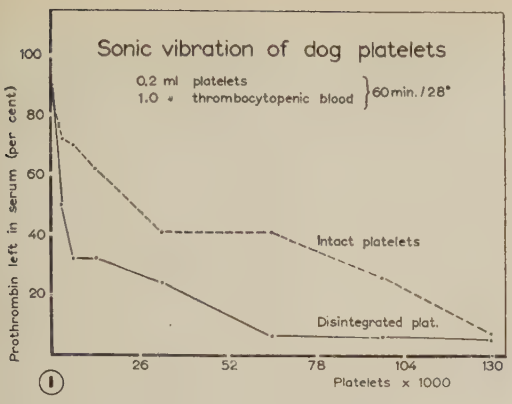
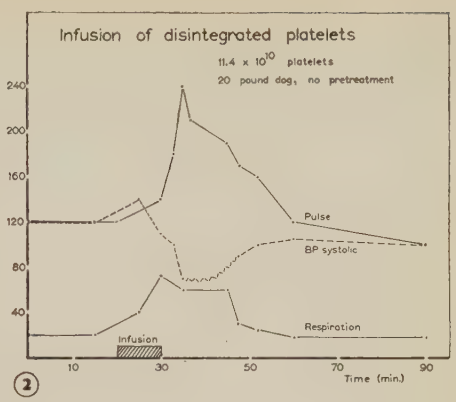
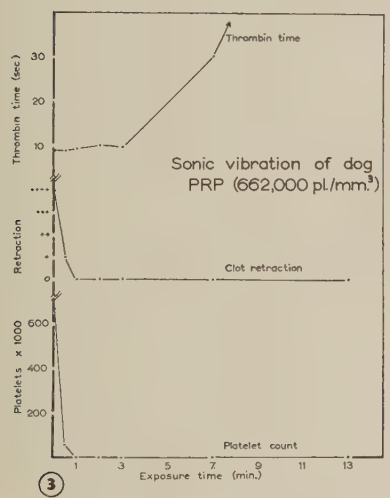
mostasis has been established(4,5). The concept of heparinemia as cause of radiation purpura has been adequately disproved(5). Transfusion of fresh platelets was first shown effective in preventing or stopping irradiation bleeding in the dog(6). The quantitative study of thrombopenic bleeding and its control was made possible by the thoracic duct cannulation technic(7) where outflow of red cells/unit time is used as index of capillary bleeding into the tissues. Irradiation, thoracic duct cannulation and appropriate coagulation studies in dogs were combined to study the 2 roles of platelets in hemostasis. We report the failure of fresh disintegrated dog platelets to correct bleeding in irradiated thrombopenic dogs.

Materials and methods. The dogs were healthy mongrels of either sex, weighing 25-30 lb. They were anesthetized with Nembutal (Abbott Lab., Chicago, Ill.) and given 500 r total body irradiation by a 250-KVP General Electric X-ray machine operated at 30 ma and filtered with 0.5 mm Cu and 1 mm Al. Tissue dose rate was 27 r/minute at TSD of 110 cm. From seventh day 75 mg daily of a serotonin analogue (1-benzyl-2-methyl-5-methoxy-tryptamine-HCl from Merck, Rahway, N. J.) were given orally. After 9-11 days the animals had less than 2,500 platelets/cu mm and a generalized purpura. Under Nembutal anesthesia the thoracic duct was then cannulated(7), 5-ml volumes of lymph were collected in tubes containing 0.8 ml 3.13% sodium citrate, and number of red cells in each portion counted (8). The dog was maintained in fluid balance by continuous infusion of saline, 5% dextrose, and Ringer solution. If lymph flow exceeded 1 ml/minute, the lymph was infused back into the dog after removing aliquots for counting. Care was taken to maintain an even flow of lymph during experiment. Each experiment consisted of 3 periods. The first was a baseline period lasting at least 4 hours, during which output of red cells in lymph, platelet count, hematocrit, and prothrombin consumption were established. The second period started with intravenous infusion of fresh, disintegrated platelets, and the effect was again followed for 4-6 hours. The third period commenced with the transfusion of fresh intcat platelets. Donor dogs were healthy mongrels of either sex, weighing 50-80 lb. Under Pentothal (Abbott) anesthesia, 400-600 ml blood were drawn from femoral artery. Blood was collected into chilled, siliconized 200-ml bottles, each containing 20 ml of 1% Na_2EDTA in 0.7% saline. Following centrifugation at 800-900 rpm (160-200 g) for 30 minutes, the supernatant platelet-rich plasmas were pooled and centrifuged again at 2,500 rpm (1,550 g) for 30 minutes. The platelets were resuspended in saline to a concentration of 1-2 millions/cu mm, and either immediately transfused or disintegrated (for 7 minutes) in 10-kc magnetostrictive oscillator (Model DF-101, Raytheon Man. Co., Waltham, Mass.). Platelets were processed as quickly as possible at 4°C. Platelets in the recipients were counted in venous blood, using phase microscope(9). Blood for prothrombin consumption was collected in dry siliconized syringes with 20-gauge siliconized needles, either from a fresh venipuncture or from an indwelling polyethylene tube in the upper vena cava. The first ml of blood was discarded, and 1 ml was then quickly placed in each of 5 acid-cleaned, dry tubes measuring 10 x 70 mm. The first contained 0.2 ml of 3.13% sodium citrate; to others, citrate was added after incubation at 28°C for 15, 30, 45, and 60 minutes. The tubes were then centrifuged at 2,000 rpm (940 g) for 5 minutes, the supernatants were diluted 1/10 in citrate dilution fluid II (10, p. 12), and prothrombin assayed in the following test system at 37°C (see 11): 0.2 ml dog brain thromboplastin (10, p. 17), 0.2 ml oxalated, adsorbed and dialyzed ox plasma (10, p. 12), 0.2 ml dog serum (10, p. 44), 0.2 ml test material, and 0.2 ml $CaCl_2$ of optimal strength. The reagents were stored at –20°C, and the system was stable during the day. On double logarithmic paper there was, within the range 5-100%, a straight-line relationship between concentration of standard dog plasma and clotting time. The system is specific for prothrombin, and the results agree closely with results of a 2-stage system (unpublished, Stormorken and Hjort). Clot retraction was observed in freshly siliconized tubes measuring 10 x 70 mm. Each tube contained 1 ml citrated platelet-free plasma from







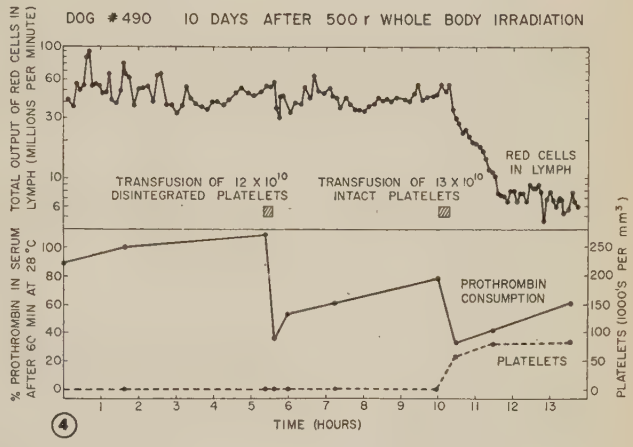


FIG. 1. Effect of platelets, intact or disintegrated, on prothrombin consumption *in vitro.*
FIG. 2. Toxic effects of disintegrated platelets.
FIG. 3. Effect of sonic vibration on platelet count, clot retraction and thrombin time.
FIG. 4. Transfusion experiment in a thrombocytopenic dog.

a thrombocytopenic dog. To this were added 0.2 ml platelet material, 0.2 ml $CaCl_2$ 50 mM, and 0.1 ml thrombin (Parke Davis Co., Detroit, Mich.), 100 NIH units/ml in 50% glycerol-saline. The tubes were incubated for 2 hours at 37°C.

Results. 1. *Effect of disintegration on platelets.* To test the effect of disintegration, dog platelets were suspended in plasma or saline and disintegrated for varying periods of time. After 3 minutes, less than 1% of platelets was still intact, and granules with a strong tendency to "agglutination" were the only remains. After 15 minutes, suspensions appeared completely empty in the phase microscope. After disintegration for 30 seconds, the platelets failed to bring about any clot retraction. Final concentration of "platelets" in the test system was 300,000 to 1 million/cu mm.

To test the effect of platelets on prothrombin consumption *in vitro,* blood was drawn from thrombocytopenic dogs having less than 2,500 platelets/cu mm. Aliquots of 1 ml were distributed in tubes containing 0.2 ml platelet material. Following incubation for 60 minutes at 28°C, citrate was added, and residual prothrombin determined. In this system disintegrated platelets were at least as active as intact platelets (Fig. 1), whether platelets were resuspended in plasma or saline. The platelet suspensions did not contain red cells on microscopical examination.

Disintegrated platelets are toxic(2,4).

When large amounts are given intravenously over a short period of time, severe circulatory and respiratory effects are regularly observed (Fig. 2). We believe that these effects are caused mainly by serotonin, and they were markedly reduced when dogs were given a serotonin analogue (12) for 4 days before transfusions.

When platelet-rich plasma was treated in the oscillator, the plasma clotting system was also affected. For instance, thrombin time increased markedly (Fig. 3). To avoid this effect in *in vivo* experiments, we resuspended the platelets in saline after second centrifugation (see above).

2. *In vivo experiments.* Fig. 4 gives results of a representative experiment. The disintegrated platelets had no effect on platelet count, nor on bleeding tendency as measured by output of red cells in lymph. However, they did temporarily normalize prothrombin consumption. Intact platelets, on the other hand, increased the platelet count to about 70% of calculated rise. Lymph started to clear during transfusion, and within 1 hour the output of red cells had dropped to about 12% of pretransfusion value. The intact platelets also increased consumption of prothrombin, and to about the same extent as did the disintegrated platelets. Similar results were obtained in 3 other dogs.

Discussion. Disintegrated platelets were studied by Axelrod(13) and our findings agree with hers. Thus, disintegrated platelets may substitute completely for intact platelets in *in vitro* clotting systems.

Clinical observations in thrombocytopenic patients have been interpreted as indicating that platelets may have hemostatic effect, even if they are not intact(2,3). The bleeding tendency following irradiation is mainly due to thrombocytopenia, and is corrected by intact platelets(4-6). Bleeding tendency in such animals may be judged clinically, but definite conclusions require quantitative experiments. Thrombocytopenic bleeding tendency may be quantitated by bleeding time, from histologic sections of lymph nodes(4-6) or by the lymph method used here. The lymph method has the particular advantage that the effect of platelets can also be *timed*. Quantitative experiments have shown that intact platelets have an immediate and dramatic effect on bleeding(4-6), while lyophilized platelets have no effect(4). Unpublished data(7) also suggest that disintegrated platelets have no effect; no clotting studies were made in these experiments.

Our results agree with these experiments and also show that disintegrated platelets did exert an adequate clotting effect *in vivo*. Therefore, failure of these platelets to stop bleeding cannot be attributed to lack of clotting effect. It might be argued that the effect of intact platelets concealed a possible late effect of the disintegrated platelets. However, the clotting effect of disintegrated platelets had already markedly decreased by the time the intact platelets were given. It might also be argued that we did not give enough of the disintegrated platelets. The quantity given, however, was about half the theoretical platelet mass of the recipient, and had a marked effect on prothrombin consumption; higher doses had undesirable side effects.

We conclude that hemostasis depends on *both* a normal clotting system *and* the mechanical action of intact platelets. The practical consequence is that non-intact platelets or biochemical platelet substitutes may not be expected to improve the bleeding tendency in thrombocytopenia.

Some of the difficulties of the experimental technic should be mentioned. Lymph flow depends on many factors: exact position of cannula, position of dog, amount and kind of fluids given, anesthesia, and finally serotonin effect of platelets. It is essential to control all these factors and maintain an even flow. Sometimes a tiny vein may carry a constant amount of blood into the duct, thus ruining the experiment. A negative effect of some test material must therefore always be confirmed by demonstration of a positive effect of intact platelets in the same dog. Finally, it is not known how many intact platelets are necessary for a positive effect, but our experience suggests a low number. Therefore, if one tests disintegrated platelets, there must be no contamination with intact platelets. On the other hand, intact platelets may be destroyed or disintegrated by prolonged storage

or handling. All these factors must be considered before conclusions are drawn.

Summary. When irradiated dogs developed severe thrombocytopenia and purpura, the thoracic duct was cannulated and output of red cells/minute counted to quantitate bleeding tendency. Fresh canine platelets disintegrated in a sonic oscillator and infused into the dogs increased prothrombin consumption towards the normal range, but had no effect on bleeding tendency. Intact platelets, however, had an immediate and dramatic effect on bleeding tendency. We conclude that a normal clotting system, including platelet factors, is not enough for normal hemostasis; the mechanical effect of intact platelets is also needed. Consequently, non-intact platelets or platelet substitutes may not be expected to improve the bleeding tendency in thrombocytopenia.

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Received April 23, 1959. P.S.E.B.M., 1959, v102.

Improvement in Nutritional Value of Peas by Cooking.* (25133)

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Numerous attempts have been made to improve the biological value of field peas (*Pisum sativum*). Woods *et al.*(1) demonstrated that protein of field peas is deficient in methionine, and that baking and autoclaving decreased growth promoting properties of the protein for rats. Peterson *et al.*(2) showed that chick growth was improved by adding methionine to diets containing peas. Our preliminary results indicated that diets containing large percentages of peas would not support maximum chick growth when methionine deficiency was corrected. Therefore, studies were undertaken to determine the influence of different methods of treating peas on growth of chicks and poults.

* Scientific Paper No. 1841, Washington Agri. Exp. Stations, Pullman. Project No. 1138.

Methods. Mature Alaska peas (*Pisum sativum*) grown in Eastern Washington were used. Cooking was accomplished by mixing ground peas with 1 to 2 parts water (approximately 30°C), spreading approximately one inch deep on trays, and autoclaving at various times and pressures (Table I). Cooked peas were immediately cooled before a fan, then cut into ½″ squares and dried in forced draft oven at 60°C. Peas were stirred after 4 hours to increase drying rate and removed from oven after 24 hours. They were then re-ground through a ⅛″ screen, and mixed in the diets. Uncooked frozen peas (var. Thomas Laxton) were partially dried in air blast, then placed in oven at 60°C to complete drying. Autoclaved peas (Table III) were prepared in same manner as cooked peas, except water

TABLE I. Effect of Cooking Peas on Poult Growth and Feed Efficiency.

Type of peas	Cooking time (min.)	Steam pressure (p.s.i.)	Ratio by wt of added H_2O:peas	Avg wt* 2 wk (g)	Feed / Gain
Corn-soy diet (control)				231b $\alpha\beta$	1.30
Mature Alaska	0	0		147e $\delta\epsilon$	1.91
Idem	0	0	1 :1	165d δ	1.69
"	15	10	1 :1	203c γ	1.43
"	15	10	1½:1	210c $\beta\gamma$	1.44
"	15	10	2 :1	211c $\beta\gamma$	1.39
"	30	10	1 :1	198c γ	1.42
"	30	10	1½:1	237ab α	1.47
"	30	10	2 :1	233b $\alpha\beta$	1.35
Mature Thomas Laxton (dry)	0	0	0	135e ϵ	2.10
Immature Thomas Laxton (fresh frozen)	0	0		205c γ	1.61
Idem	30	5		252a α	1.34

* Latin letters after weight in this column refer to significance at 5% level, and Greek letters refer to significance at 1% level. Treatments with a common letter are not significantly different at level indicated. Treatments without a common letter are significantly different at level indicated.

was not added. Day-old Broad Breasted Bronze poults or New Hampshire chicks were randomly distributed into pens of 10 birds each and given feed and water *ad lib*. Poults and chicks were maintained in electrically heated batteries with raised wire floors. Each experimental diet was fed to 3 groups of birds. A 28% protein basal poult diet of following percentage compositions was used: 77.10 peas (24% protein), 12 herring fishmeal, 3 dehydrated alfalfa meal, 4 dicalcium phosphate, 2 limestone, .5 salt, .6 DL-methionine, and vitamin mix supplying 5300 I.U. Vit. A, 1100 I.C.U. Vit. D, 6.6 mg riboflavin, 15.4 mg pantothenic acid, 1.65 g choline chloride, .11 mg folic acid, 22 mg niacin, 11 mg procaine penicillin, 11 I.U. Vit. E, and .2 g manganese sulfate/kilo of diet. The control diet was 48.6% corn and 28.5% dehulled soybean oil meal, replacing peas. A 23.4% protein basal chick diet of following percentage composition was used: 93.4 peas (24% protein), 2 tallow, 1.5 steamed bonemeal, 1.5 limestone, .5 salt, 16 DL-methionine, .02 manganese sulfate and vitamin mix supplying 2640 I.U. Vit. A, 440 I.C.U. Vit. D, 2.86 mg riboflavin, 9.24 mg pantothenic acid, 880 mg choline chloride, 11 mg procaine penicillin, 26.4 mg niacin, 2.86 mg pyridoxin-HCl, .088 mg biotin, .0088 mg Vit. B_{12}, .55 mg folic acid, 1.76 mg thiamine-HCl, 22 I.U. Vit. E, and .396 mg menadione bisulfite/kilo of diet. Control diet was 56.8% corn and 36.6% dehulled soybean oil meal, replacing peas. Separation of peas into 2 fractions (Table IV) was accomplished by stirring 1 kilo of finely powdered peas into 5 kilo of 0.6% NaOH, adjusting to pH 11.3 and centrifuging. The solid residue (alkali-insoluble peas) was air dried with fans and ground before mixing into diets. The alkali-soluble portion was adjusted to pH 5.5 with concentrated HCl and centrifuged. The precipitate (alkali-soluble peas) was treated as above before mixing into diets. Alkali-soluble fraction contained 64% and the alkali-insoluble fraction contained 7% protein (Kjeldahl).

Results. Results of 4 experiments are reported. Cooking of both mature Alaska peas and fresh frozen Thomas Laxton peas markedly improved growth rate and efficiency of feed utilization in turkeys (Table I). A greater improvement was obtained by cooking peas 30 minutes as contrasted to 15 minutes, and by using a ratio of water to peas of 1½ : 1 and higher. Results comparable to control diet were obtained by cooking peas 30 minutes with higher water-to-peas ratio. Immature peas (var. Thomas Laxton) were much superior to mature peas when compared on uncooked basis.

Growth, feed efficiency, and dry matter retention (determined by difference between dry matter ingested and dry matter eliminated) of chicks were markedly improved by

TABLE II. Effect of Cooking Peas and of Added Tallow on Performance of Chicks.

Tallow (%)	Peas in diet (%) Raw	Cooked	Soybean oil meal (%)	Productive energy (cal/g)	Avg wt 4 wk (g)	Feed / Gain	Dry matter retention 4th wk (%)
Corn-soy control diet				2.16	347	1.87	70
2	93.4			1.96	227	2.66	55
2		93.4		2.02*	366	2.00	64
5	85.0		6	2.07	238	2.10	
5		85.0	6	2.12	340	1.77	
20	52.0		24	2.56	289	1.82	

* Fraps' productive energy value for cooked peas is 1.917 cal/g, and for raw peas is 1.954 cal/g.

cooking peas (Table II). Although increasing the productive energy content of diets by adding tallow brought about some improvement in growth and feed efficiency, the improvement was not as great as by simply cooking the peas. Autoclaving peas also improved both the growth and efficiency of feed utilization in chicks (Table III). Diet containing alkali-soluble fraction supported significantly ($P<.01$) better growth than diet containing alkali-insoluble fraction. Raw pea protein readily extracted by dilute alkali was high in feeding value.

The reason for nutritional improvement of peas by autoclaving is unknown. That it is not simply a physical change in peas, making more energy available to the animal, or improving protein quality, is shown by results presented in Tables II and IV, respectively. Increasing the caloric content of pea diets by addition of tallow did not give as great a response as the cooking treatment, even though calculated energy content of the diet was greatly increased.

Perhaps the best explanation is that heat treatment inactivates a growth inhibitor or toxic substance in peas. Several growth inhibitors for chicks have been demonstrated in various plant materials(3,4,5,6). One of these, a trypsin inhibitor found in raw beans, is inactivated by heating in presence of moisture. Borchers *et al.*(7), however, did not detect trypsin inhibitor in peas. Another member of the pea family, *Lathryus odoratus*, contains a toxic substance for animals, B(r-L-glutamyl) aminopropionitrile(8), which was not inactivated by cooking(9) or heating(10).

Armbruster and Murray(11) observed that cooked peas were not superior to raw peas for weanling rats. Our recent experiments showed that cooking peas slightly depressed female rat growth, but significantly ($P<.01$) increased male rat growth. No such difference between sexes have been noted with chicks or poults fed cooked peas. The reason for differences in response of chicks and rats to cooking of peas is not known.

TABLE III. Effect of Autoclaving of Peas *(Pisum sativum)* on Growth of Chicks.

Diet	Avg wt* 3 wk (g)	Feed / Gain
Corn-soy (control diet)	223a α	1.72
Raw peas	161b β	2.30
Cooked peas, 30 min.	230a α	1.87
Autoclaved, 30 "	229a α	1.86

* See footnote, Table I, for explanation of letters in column.

TABLE IV. Effect of Alkali-Soluble and Insoluble Fractions of Peas on Chick Growth.

Diet	Avg wt* 3 wk (g)	Feed / Gain
Positive control (corn-soy)	213b α	1.77
Raw peas	148d γ	2.66
Autoclaved peas	227ab α	1.81
Alkali-soluble peas†	231a α	1.79
" -insoluble " ‡	185c β	1.94

* See footnote, Table I, for explanation of letters in column.

† Contained 16.4% alkali-soluble fraction (64% protein), and 26.12% sucrose replacing autoclaved peas in autoclaved pea diet.

‡ Contained 74.4% alkali-insoluble pea fraction (7% protein) and 19.0% isolated soybean protein (Archer-Daniels-Midland Assay C-1), replacing 93.4% peas in diet.

Summary. Growth and feed utilization of chicks and poults fed diets containing high levels of peas (*P. sativum*) were markedly improved by cooking or autoclaving peas prior to mixing into diets. Greatly increasing cal-

oric content of diets by adding tallow did not give a growth response comparable to that obtained by simply cooking the peas. Alkali-insoluble fractions of peas depressed chick growth but alkali-soluble fractions did not. It is suggested that peas (*P. sativum*) contain a growth inhibitor for birds that is inactivated by heating treatments employed.

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Received April 27, 1959. P.S.E.B.M., 1959, v102.

Electrophoretic Migration of Serum Lipoproteins in Starch Gel.* (25134)

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Electrophoresis in starch gel(1), is being increasingly used mainly because of its good resolution of protein fractions. During our studies of serum and cerebrospinal fluid proteins(2) with this method we found it advantageous to stain half of the gel electrophoretogram for lipoproteins. Oil red O was selected as coloring agent in staining the lipoproteins in starch gel as described by Smithies (personal communication). Initially considerable difficulties were encountered with reproducibility of the pattern using this method; later, however, we determined that these variations were mostly produced by inconstant composition of commercial oil red O which contains components that stain albumin and globulins as well as lipids. Partial purification has reduced these inconsistencies and has enabled relatively constant lipoprotein patterns in the starch gel to be obtained. To interpret the patterns obtained with oil red O, we thought it important to identify the bands that were seen. Serum lipoproteins were fractionated by starch granule electrophoresis and by sedimentation in the preparative ultracentrifuge. The isolated fractions were submitted to electrophoresis in starch gel and the results described below.

Materials and methods. Human serum was separated by centrifugation, pooled and stored at 4°C no longer than 48 hours before ultracentrifugation or electrophoresis. All chemicals were of reagent grade and the solvents were redistilled. Double glass-distilled water was used in all experiments to reduce contact of lipoproteins with copper ions. Starch gel electrophoresis was carried out as described by Smithies(1). Coloring of lipoproteins with oil red O was performed as previously reported(2) and crude oil red O was partially purified eliminating the protein staining components with a technic we developed(3). Spinco Model L preparative ultracentrifuge was used to prepare high and low density lipo-

* This investigation supported by special clinical traineeship from Nat. Inst. of Neurol. Dis. and Blindness, U.S.P.H.S.; and from Nat. Multiple Sclerosis Soc.

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proteins. Specific gravity of serum was adjusted to 1.063 g/ml with a saturated solution of KBr and $NaNO_3$ in equal parts(4). In Rotor 40 the specimen was spun at 105.400 x G for 24 hours. Floating lipoproteins were collected and designated as low density lipoproteins. The bottom of the 1.063 tubes was aspirated and adjusted to specific gravity of 1.21 g/ml and centrifugation repeated. Floating lipoproteins were collected and designated as high density lipoproteins. Alpha-1, alpha-2 and beta lipoproteins were obtained by starch granule electrophoresis. The starch blocks were prepared as described by Kunkel(5), with minor modifications. Barbital buffer of pH 8.6 and ionic strength of 0.06 was used. After completion of electrophoretic separation, the block was cut in segments, suspended in 0.9% saline and a protein curve was prepared by the method of Folin-Ciocalteu. Tubes of beta, alpha-2, and alpha-1 (containing a large proportion of albumin) zones were pooled into the 3 respective fractions and concentrated by negative pressure dialysis in collodion bags according to the method of Mies(6). The concentrated lipoprotein fractions were then subjected to electrophoresis in starch gels. The strips were stained with purified oil red O and scanned with a recording photoelectric densitometer.‡

In another block, segments were extracted with methylal : methanol 4 : 1 mixture and phospholipid and cholesterol analyses were made by methods of Fiske and Subbarow and Sperry and Schonheimer respectively, to determine the position of lipoproteins in the starch block.

Results. In starch gel electrophoresis, the beta lipoproteins prepared by starch granule electrophoresis migrated close to the origin forming a wide band, which spread from the origin toward the anode forming a plateau (Fig. 1 A). The alpha-2 lipoproteins also remained close to the origin similar to the pattern of beta lipoproteins; however, there was a rising front present towards the anode (Fig. 1 B). The alpha-1 lipoproteins migrated in the postalbumin area as a band, which had a rapidly rising limb and a somewhat gradually falling limb towards the anode (Fig. 1 C). This asymmetrical leading edge of the alpha-1 component was occasionally resolved into an additional band.

To confirm the identity of lipoproteins eluted from the starch block and rule out the possibility that the beta lipoproteins might have extended into the area of alpha-2, the following experiment was performed. All tubes of a starch block were separately concentrated and submitted to electrophoresis in starch gel. The slow lipoprotein band was seen in concentrates from both beta and alpha-2 segments and little, if any, lipid-containing material was seen in segments that constituted the valleys between peaks of starch block electrophoresis. The fast band was found in the concentrate from albumin-alpha-1 segments. These patterns were in agreement with the phospholipid curve from a block of the same serum (Fig. 2).

Low density lipoproteins prepared by ultracentrifugation remained close to the origin as did the beta lipoproteins from the starch block, but they also showed a rising front towards anode similar to the alpha-2 lipoproteins. The high density lipoproteins showed a band similar in shape and position to that given by alpha-1 lipoproteins eluted from the starch block.

When whole serum was applied, patterns were obtained which showed a band close to the origin in the position of the low density lipoproteins and another band in the postalbumin region, where the high density lipoproteins migrated (Fig. 1 D). It was noted that the band close to the origin showed considerable variations among individual patients. This band also decreased in intensity more rapidly than the faster band when serum was stored, and repeated runs performed or when concentration by lyophilization rather than pressure dialysis was employed.

Before purification of oil red O was undertaken, the number of bands seen on the gel strips where whole serum has been separated, was not constant and at times all serum proteins were stained. Some bands in addition appeared to be reddish-brown or even yellowish-brown. This occurred whenever a new

‡ Generously supplied by Airborne Instrument Co., Mineola, L. I., N. Y.

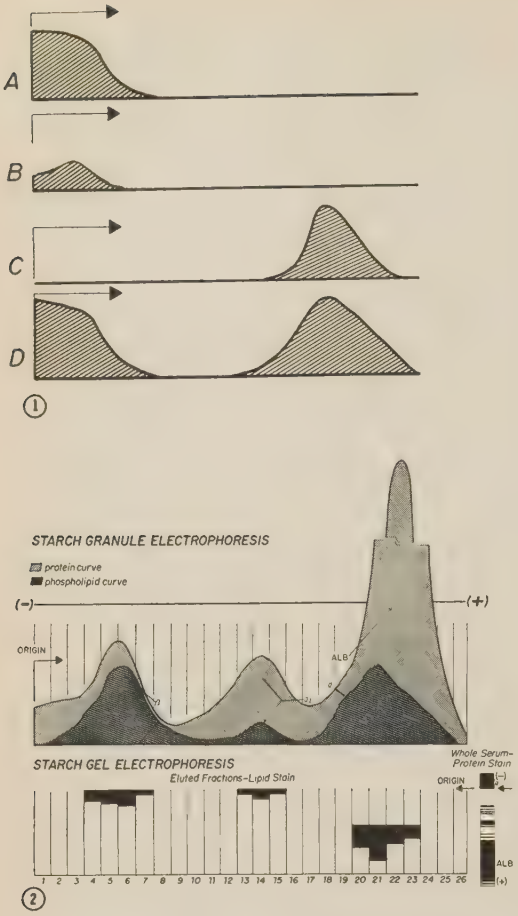

FIG. 1. Photoelectric scan patterns of starch gel electrophoresis strips. A. Beta lipoprotein; B. Alpha-2 lipoprotein; C. Alpha-1 lipoprotein; D. Full serum lipoprotein pattern. Arrow marks point of application and points toward anode.

FIG. 2. Comparison of migration of serum lipoproteins by starch granule electrophoresis and starch gel electrophoresis. Protein curve (lightly shaded) and phospholipid curve (heavily shaded) of starch granule electrophoresis are shown in upper part of Figure. Twenty-six segments were eluted and run in starch gel. Location of lipoprotein bands in starch gel is illustrated in lower part of Figure. Gamma section of starch granule block has been omitted.

batch of staining solution was prepared, which happened to contain a large amount of yellow-brown protein staining components. Following use of purified oil red O, only the slow and fast bands were consistently seen.

Discussion. Electrophoretic mobilities of alpha-1 and beta lipoproteins in starch gel could be anticipated. They were similar to zone electrophoresis patterns obtained with other supporting media such as filter paper, starch granules or agar, and confirm the findings of Silberman(7) and Poulik and Smithies (8). The behavior of alpha-2 lipoproteins, however, is of interest. The work of Kunkel and Trautman(4) indicated that some alpha-2 lipoproteins are lighter than serum and possibly contain chylomicra. Our results tend to support that evidence, in that migration of alpha-2 lipoproteins in starch gel is similar to the beta, low density, lipoproteins. It appears therefore that when whole serum is submitted to starch gel electrophoresis, the slow band includes beta and alpha-2 lipoproteins, whereas the band in the postalbumin region contains only high density lipoproteins.

When fat dyes are used which contain protein staining components for demonstration of lipoproteins, the starch gel method offers some advantages over paper electrophoresis. Lipoproteins in gel migrate in areas, where no major protein component is present and the co-staining of proteins is therefore less disturbing in the interpretation of patterns. The major disadvantage of the gel method is that quantitative estimates from gel strips can be obtained only by scanning devices and these are less accurate than data obtainable with dye elution technics.

Summary and conclusions. 1. Alpha-2 and beta lipoproteins in starch gel migrated close to the origin, whereas alpha-1 lipoproteins migrated in the postalbumin region. 2. Some of the causes of inconsistencies of lipoprotein patterns in starch gel are discussed.

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Received May 8, 1959. P.S.E.B.M., 1959, v102.

General Character of Epidermal Papillomas Induced by Carcinogens on Mouse Skin as Disclosed by Transplantation. (25135)

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The epidermal papillomas arising on mouse skin exposed to tar or other chemical carcinogens are the most common of induced cutaneous growths, yet they have only of late been propagated(1). Those chosen for transplantation, as most likely to succeed, had persisted long after tarring; and they were transferred to deep sites in sucklings and weanlings. Six out of 8 of them grew progressively in both and were maintained in series. But the question arises whether they were representative of the generality of papillomas; for when situated on the skin these are protean in form, differ much in vigor, and nearly always disappear eventually unless stimulated by promoting agents. For these reasons it has seemed possible that they might be of several sorts. To learn whether this is the case many have now been transferred and studied.

Method. The tumors were induced in C strain mice, as previously, and with the same "Landsteiner tar,"—which greatly promotes the growth of papillomas. They were transplanted by trochar to subcutaneous situations in sucklings and weanlings, just as before. The tar was applied once a week to large areas on the back of numerous adult mice of both sexes until the first papilloma appeared, but only after longer intervals later, to lessen inflammation and bacterial infection; and the growths were transferred when large enough to provide tissue for several to many implantations. They then showed the usual diversity of form, some being broad-based or onion shaped or pedunculated, whereas others were as yet mere unerupted mounds that were papillomatous, as made plain by their palisade markings on vertical incision. The stained sections made of them, when enough tissue remained for this, validated their character.

Results. Most of the papillomas transferred were the first to have arisen as result of the tarrings and such growths are especially liable to undergo regression *in situ* if exposure to the carcinogen inducing them is discontinued soon after they have appeared(2). Nevertheless they did as well in their new hosts as those that happened to be second to fifth in order of appearance on the tarred skin. Five out of the total 29 failed to yield growths on transfer, one of them because of purulent infection. Eight gave rise to carcinomas only, although when implanted they had looked in the gross like typical papillomas; but sections made later of 5 of them showed cancer to have been present as well. Three yielded growths of mingled character or gave rise to carcinomas in some animals and papillomas in others. Thirteen produced papillomas only (benign keratomas(1)). Cancers often derived from these after further transfer.

Every papilloma successfully transferred was passaged in order to learn more about it. Nearly all of the numerous growths thus obtained enlarged progressively and after a few months proved fatal, but very occasionally a small one stopped growing because it had keratinized completely. All of them proved to be of the same kind as the 6 originally propagated, and they manifested the same individual differences in capability. Eight out of 16 (counting the 3 in which a carcinomatous element was present) proved to be "able-bodied" papillomas (Type A(1)). They consisted of neoplastic cells which had retained the ability of normal epidermis to extend laterally on the bare wall of the graft pocket and to differentiate inwards, forming squamous, keratinized elements. These activities resulted in gradually enlarging cysts lined with a thin layer of papillomatous tissue and full of a dense mass of keratin. The other 8 papillomas were composed of cells that were crippled to a greater or less extent, both in ability to keratinize and to extend on the bare wall of the pocket in which they lay (Type C). These growths failed, more or less completely, to line the pocket, and they formed less keratin than the "A" growths. But the keratin acted as an irritant on the con-

nective tissue directly exposed to it, and the result was a cyst containing thin fluid with keratin suspended in it. The cyst had walls that were bare except for a papillomatous mound projecting into its interior, or a cauliflower, sometimes low and broad-based but often pedunculated and not infrequently almost entirely filling the cyst cavity. All gradations between A and C tumors occurred, and their cells did not differ significantly in aspect. Those of both growths underwent carcinomatous change about equally often. As a rule the A papillomas extended more rapidly on the walls of the enlarging cysts than did the cancers arising from them, and these latter could be left behind by a judicious selection of grafts at the next transfer. The C papillomas on the other hand failed to outstrip the cancers and hence were soon supplanted by them.

Dr. James S. Henderson of this laboratory has generously permitted a report here on the character of 12 epidermal papillomas he has recently propagated. Four were induced by tarring the backs of C mice and 8 by the repeated application of 20-methylcholanthrene in benzene or Crabtree's medium(3). All proved on transplantation to be of the kind just described and those of the A and C types were equally frequent.

Accident, infection of the grafts, or some host influence may have determined the failure of the papillomas that gave rise to no tumors after transfer. The many that succeeded have yielded growths that enlarged at much the same moderate pace,—a surprising fact in view of the activity that some papillomas manifest when situated on the skin and the indolence of others. Evidently local cutaneous conditions have much to do with this difference.

Conclusions. The transplantation of numerous epidermal papillomas induced by tar or 20-methylcholanthrene has shown them to be due to neoplastic changes of a single kind. Their wide diversity of form and behavior, when situated on the skin, seems referable in the main to differing capabilities of their cells. The great majority of them, indeed perhaps all, are transplantable in series, when favoring sites are provided them.

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Received May 11, 1959. P.S.E.B.M., 1959, v102.

Experiments on Cholesterol Atherosclerosis in Rabbits.* (25136)

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Duff(1) suggested that atherosclerotic lesions may be due to impurities when rabbits are fed cholesterol over a long period. Recently Altschul showed that heated cholesterol (2) or dried and heated egg yolk(3) were more effective than cholesterol itself in obtaining atherosclerotic damage in rabbits, guinea pigs or hamsters, suggesting that companions of cholesterol or products formed during heating were responsible. Nevertheless, most investigations on cholesterol atherosclerosis were made with commercially available, so-called pure cholesterol† which contained small amounts of impurities; (cf. 4). Experi-

* Our work was supported by U.S.P.H.S. Grant, Institutional Grant from Am. Cancer Soc., and Jane Coffin Childs Memorial Fund.

† Anitschkow and Chalatow(5) and Wacker and Hueck(6) reported feeding "pure" cholesterol ("reines Cholesterin") to rabbits. Study of original papers suggests that they really used commercial cholesterol. Their work, therefore, is not as conclusive as appears from Kritchevsky's book(7).

ments therefore seemed advisable with carefully purified cholesterol and with impurities contained in commercial, so-called pure cholesterol. Usual methods do not completely purify cholesterol. Only conversion to 5,6-dibromocholestanol-3 and subsequent debromination gave pure material(8). Later, however, L. L. Smith(9) found that even chromatographically pure 5,6-dibromocholestanol-3 gave partially autoxidized cholesterol. In the present work these difficulties have been overcome.

Methods. Preparation of pure cholesterol. From 250-300 g batches of commercial cholesterol (Wilson) the oxalic acid complex was prepared in ethylacetate(10) and after its decomposition the cholesterol was dried in air. Every day 15 g of this cholesterol were brominated for final purification according to Fieser(11), but the appropriately washed last ether solution was diluted with about two-thirds its volume of 95% ethanol and the solvents distilled off *in vacuo*. When cholesterol started to crystallize the flask was cooled in ice. The crystals were filtered with suction and washed with ice-cold ethanol. As soon as most of the solvent was sucked off, a rubber dam was applied and suction continued for one-half hour. Ten to 12 g pure cholesterol were obtained, with about 7-8% moisture (ethanol). This figure was used to calculate amount for feeding. When 1.5 g of a C^{14}-cholesterol of 1300 c/min/mg were treated in exactly the same way a reverse phase chromatogram on kerosinized paper(12) showed only one peak of radioactivity. A similarly treated sample gave only one single peak on a silica gel column. *Isolation of impurities from commercial cholesterol.* 200 lb of commercial cholesterol (Wilson) were purified through the courtesy of Schering Corp., Bloomfield, N. J., by treatment with anhydrous oxalic acid in hexane. The mother liquors were distilled leaving impurities as a brown residue (about 2% of starting material). Three hundred g of this were treated in ethylacetate with 60 g anhydrous oxalic acid, the complex discarded and the mother liquor boiled in a large beaker with water until ethylacetate was eliminated. The air-dry residue contained 14% cholesterol-like substances, determined as digitonides. It was used together with commercial cholesterol to feed Group IV rabbits. For Group III rabbits, which received impurities alone, the insoluble residue was dissolved in 95% ethanol and precipitated with digitonin, the mother liquor evaporated *in vacuo* and last traces of water eliminated azeotropically with benzene. The dry residue was taken up in pentane, filtered from the insoluble and evaporated to dryness. The soft air-dried residue still contained 7.8% digitonin precipitable material. Two to 3 months old Dutch belted, New Zealand and Chinchilla rabbits were so arranged that each of 5 groups contained all 3 types of animals and members of both sexes. Ten animals were started in each group, but some died of respiratory infections (Table I). Every morning each animal was fed 50 g rabbit chow with 5% cotton seed oil and the test substance. When this was eaten, normal rabbit chow, hay, carrots and lettuce were given. Test substances were fed for 12 weeks, omitting Sundays. Each rabbit in Group I received/day 900 mg of purified cholesterol, Group II got 900 mg of commercial cholesterol (Wilson)/day and Group III had 200 mg of impurities/day containing 15.6 mg of digitonin precipitable material. Group IV received 200 mg of impurities/day, containing 28 mg digitonin-precipitable substance (calculated as cholesterol) plus 872 mg of commercial cholesterol. In this mixture impurities amounted to about 16%. Group V were control animals which received only 5% cotton seed oil with their daily diet. For Groups II-IV the cholesterol and/or its impurities were dissolved in ether, but to exclude ether peroxides the purified cholesterol for Group I was dissolved in pentane before added to the chow. From the combined aortae of each group, cholesterol was isolated after hydrolysis. For serum cholesterol determinations 5 ml blood were withdrawn from each rabbit by heart puncture. In Groups I and II the method of Kingsley and Schaffert(13) was used, but unfortunately no smaller aliquots were made and the values obtained are therefore too low and cannot be statistically evaluated. For Groups III-V the procedure of Abell *et al.*(14) was employed. *Histological procedures.* Animals were killed by cervical

TABLE I. Effects of Purified and of Commercial Cholesterol and Its Impurities on Serum and Aorta Cholesterol and the Degree of Atherosclerotic Lesions.

Group No.	Material added to normal diet	Total mg cholesterol fed/animal /day	No. of animals		Cholesterol in 100 ml serum		mg/g wet aorta	No. of animals with atherosclerotic lesions				
			At start	At end	mg	Mean ± S.E.		5+	4+	3+	2+	+	—
I	Purified cholesterol	900	10	10	488*		5.0	5	2	2	1	0	
II	Commercial cholesterol	"	"	5	<488*		6.4		2	3	3	2	
				5	>455	455 ± 0							
III	Impurities	15.6	"	9	25– 258	100 ± 28.4	.9						9
IV	Commercial cholesterol + impurities	872 28	"	6	400–4000	1947 ±557	5.4		1		1	4	
V	Controls	0	"	7	14– 55	24 ± 5.4	.9						7

* Precise values not determined. However, in 40 animals receiving 900 mg commercial cholesterol/day for 12 wk in other experiments, a mean was found of 804 mg (120-1980, ± 72).

fracture and tissues fixed as usual for sectioning. Hematoxylin-eosin was used for staining. Sections of aorta, tongue, heart, kidney, adrenal, liver, spleen and eye were coded for grading based on nature, frequency and intensity of atherosclerotic lesions in each section. From this the grade of damage for each animal was compiled. The strongest alterations were assigned as 5+, absence of damage as –, with 4+, 3+, 2+, + as intermediates.

Results. Table I shows that in Groups I, II and IV feeding of large amounts of cholesterol increased serum cholesterol and that cholesterol laid down in the aortae was increased about 5 to 6 times. Feeding a large amount of impurities from commercial cholesterol in Group III did not influence aorta cholesterol, but the small amount of digitonin precipitable substance left in the impurities increased serum values to about 4 times the normal.

The last columns of Table I present histological investigation of the tissues. Extensive damage occurred in Groups I and II, but highly purified cholesterol caused more severe lesions in Group I. Animals in Group III, fed a large amount of impurities had normal aorta cholesterol and showed no lesions although their serum cholesterol was somewhat higher than normal. A single animal had some calcification of the aortic media. Animals in Group IV which received the same total amount of cholesterol as those of Groups I or II but simultaneously a large amount of the same impurities as Group III had high aorta cholesterol and serum cholesterols at least as high as in Groups I and II, but damage from atherosclerotic lesions in their tissues was considerably less. Appropriate calculation shows that the differences between control and experimental groups for serum cholesterol and for the atherosclerotic lesions are statistically significant. It appears therefore that natural impurities of cholesterol include substances which counteract the effect of pure cholesterol in producing atherosclerotic lesions.

Little is known about the chemical nature of these substances. Cholesterol, made mainly from spinal cords of cattle and from cattle or sheep brain, is probably deposited from blood and should therefore contain companions simi-

lar to those isolated from blood and other tissues. Such investigations by Hardegger, Ruzicka and Tagmann(15) showed that the substances from non-saponifiable extractives of aorta, serum, liver and other tissues are either oxidation products of cholesterol or derivatives of those. This is understandable, considering experiments of Borgstroem and Wintersteiner(16) which show how readily cholesterol can be oxidized by air under conditions similar to the situation of cholesterol in blood, where it is exposed to dissolved oxygen in the presence of solubilizing substances and buffers at a slightly basic pH. More recent contributions to the problem of companions of cholesterol have been made by Fieser(11) and Schwenk *et al.*(17). The impurities may include unknown substances other than steroids. The natural companions of cholesterol, contained in the material used in this work, are evidently different from substances formed when cholesterol is heated(2,3). Such substances enhance the atherosclerotic effect of cholesterol, but the same author(12,18) has found that *in vitro* oxidation of cholesterol decreases its atherogenicity.

Summary. Feeding of highly purified cholesterol to rabbits for 12 weeks resulted in high serum cholesterol and atherosclerotic lesions, which appear more severe than those obtained with commercial cholesterol. This latter contained about 2% impurities isolated from mother liquors after treatment of commercial cholesterol with anhydrous oxalic acid in organic solvents. Such impurities did not produce atherosclerotic lesions when fed to rabbits, but when given together with cholesterol caused less extensive lesions than those obtained with pure or commercial cholesterol.

The help of Erszebet Joachim, Eva Moring and Deirdre McLeod is gratefully acknowledged.

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Received May 13, 1959. P.S.E.B.M., 1959, v102.

In vitro Absorption of Serotonin by Thrombocytes of Rheumatoid Arthritic and Non-Arthritic Individuals.* (25137)

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It was noted(1) that the 5-hydroxytryptamine (5-HT, serotonin) content/unit of circulating blood thrombocytes was decreased in patients with rheumatoid arthritis or other inflammatory states, as compared to patients with no evidence of inflammation. Since Spector and Willoughby(2) have shown that 5-HT

* Supported by grants from Nat. Inst. of Arthritis and Metab. Dis., N.I.H., U.S.P.H.S., and Am. Heart Assoc.

appears extravascularly early in evolution of the inflammatory process, it was thought that thrombocyte damage might have occurred and that the decreased 5-HT might simply mean that 5-HT is restored to blood less quickly than are thrombocytes, or it might be conjectured that release of 5-HT from intact thrombocytes had in some way been triggered without equal restoration. However it seemed desirable to look at groups of patients with and without inflammatory states and with rheumatoid arthritis to test the ability of their thrombocytes to absorb 5-HT *in vitro* under standard imposed conditions. There was a possibly significant decrease in amount of 5-HT picked up by thrombocytes from blood of patients with non-rheumatoid inflammatory diseases and rheumatoid arthritis as compared to individuals with no demonstrable inflammation.

Methods. Three groups of patients were studied. These were as previously defined (3) and consisted essentially of Group A with no evidence of inflammatory disease, Group B with significant but non-rheumatoid inflammatory processes,† and Group C with unequivocal active rheumatoid arthritis. Blood was obtained by venipuncture and thrombocyte-rich plasma harvested by the method of Dillard, Brecher and Cronkite(4). Blood volume of total sample was measured by marking the blood level on each tube and finally filling the emptied tubes with water, measuring water volume and correcting for known volume of added anticoagulant; total volume of thrombocyte-rich plasma obtained from the sample was also recorded.‡ Four 2-ml aliquots of thrombocyte-rich plasma were then added to 2 sets of duplicate siliconed 25 ml Erlenmeyer flasks containing, respectively, 0.3 ml of 1% disodium ethylenediaminotetroacetate (EDTA) in 0.7% sodium chloride and either 3 ml (Set A) or 2 ml (Set B) of physiologic saline.§ At zero time, exactly 1 ml of physiologic saline containing 1 μg‖ of serotonin creatinine sulfate (furnished kindly by Abbott Labs) was added rapidly to flask B and immediately immersed in Warburg shaker water bath at 37°C. At exactly 6 minutes,‖ each flask was instantly iced by rapid immersion in ice bath. Contents of each flask were transferred quantitatively with iced saline wash to iced siliconed tubes. The tubes were then centrifuged at 5°C, 500 g, for 30 minutes. Thrombocyte buttons were drained carefully, and walls of tubes were wiped dry. Thrombocytes were then resuspended smoothly and disrupted in 3 ml of 0.14 sodium chloride in 0.02 M sodium hydroxide and subjected as in previous studies(1) to 5-HT extraction and measurement, a standard curve being carried through each extraction procedure as previously. Thrombocyte protein content was es-

† For the first time, due to chance population of hospital wards during the study, Group B series included predominantly acute inflammatory processes particularly lobar pneumonia, whereas prior series included a large proportion of chronic inflammatory processes, e.g. chronic lung disease with secondary chronic bacterial infection.

‡ From this point on, both preparation of platelets for biuret determination and their preparation for 5-HT measurement differed from (1); hence absolute data of present study cannot be compared directly with data of (1).

§ In the study summarized in Table III only, the procedure was varied so that thrombocytes were separated from platelet-rich plasma by centrifuging at 500 g for 30 minutes at 5° C and resuspending in physiologic saline; 2 ml aliquots of saline suspension were then used in each flask, plus 0.3 ml of EDTA and 2 ml of platelet-free autologous or homologous plasma, as indicated in Table III, and in each flask of Set A 1 ml of saline. By this means the effect on recovery systems of inflammatory plasma on noninflammatory thrombocytes and of noninflammatory plasma on inflammatory thrombocytes was observed.

‖ Despite obvious disadvantage of working in the range of a relatively steep slope on curve(5), the information desired concerned ability of thrombocytes to absorb small amounts of 5-HT rapidly. Amount of 5-HT added and test period were constants selected after considerable number of preliminary tests covering periods from 1 to 30 minutes, 6 minutes was selected as time sufficient for few normal thrombocyte-rich plasma samples (prepared as and in amounts specified) to absorb entire added 1 μg of serotonin creatinine sulfate. Disadvantage of the relatively steep slope of curve was minimized by meticulous attention to and care in timing of interval available for absorption of added 5-HT by the thrombocytes. Absorption of 5-HT at low temperatures is much decreased(5) from absorption at 37° C.

TABLE I. Spontaneous Levels of 5-HT in Human Thrombocytes Used in Absorption Experiments of Table II.*

Group (No. of subjects)	Per thrombocytes from 100 ml blood: mg protein	Per thrombocytes from 100 ml blood: μg 5-HT	Per 100 mg of thrombocyte protein, μg 5-HT
A. Noninflammatory (11)	*34.5 ± 13.4*†	*14.4 ± 6.1*	*41.3 ± 12.8*
B. Inflammatory (9)	*47.4 ± 21.5*	*12.7 ± 8.3*	*27.6 ± 13.7*
t, p (*vs* Group A)	1.645, >.05	.515, >.05	2.306, <.04
C. Rheumatoid (11)	*50.2 ± 12.6*	*13.7 ± 6.6*	*27.2 ± 12.0*
t, p (*vs* Group A)	2.841, <.012	.250, >.05	2.665, <.02
" (" " B)	.374, >.05	.294, >.05	.075, >.05

* As noted in footnote (see *Methods*), a difference both in technical methods used and in composition of Group B precludes direct comparison with a previous report(1). Although both (1) and the present work were designed solely for comparison of groups within the individual study, the above data on thrombocyte proteins have absolute validity (because of a washing step introduced) in contrast to(1) where only comparative validity was sought.

† S.D.

timated as before(1) by biuret determination.¶ A 0.7 ml aliquot of thrombocyte-rich plasma was centrifuged at 1000 g for 15 minutes at 5°C, the thrombocytes resuspended and washed in 5 ml saline, recentrifuged and resuspended to original volume in demineralized water for the determination.

Results. Table I summarizes data concerning overall amounts of spontaneous 5-HT content of human thrombocytes, expressed both/unit of blood and/unit of thrombocytes. As in previous studies(1), the amount of 5-HT/unit of blood was uniform in the 3 groups. Amount of thrombocyte protein/unit of blood was increased above noninflammatory Group A levels in the rheumatoid Group C, however, so that the 5-HT content/unit of thrombocytes was decreased ($p<.02$). A corresponding decrease in the inflammatory Group B approached but did not reach significance ($p<.04$). This was in contrast to corresponding findings in (1) where the inflammatory Group B also was decreased ($p<.02$) and may reflect the inclusion of more acute inflammatory processes in Group B of present series, as previously noted.

Table II summarizes data which constituted the chief objective. Expressed as serotonin creatinine sulfate (in contrast to Table I data), column 2 of data suggests that throm-

TABLE II. Absorption by Thrombocytes of Serotonin Creatinine Sulfate Added to Thrombocyte-Rich Plasma.

Group (No. of subjects)	mg thrombocyte protein/2 ml thrombocyte-rich plasma aliquot	μg serotonin creatinine sulfate absorbed, of 1 μg added to 2 ml thrombocyte-rich plasma aliquot
A. Noninflammatory (11)	*1.84 ± .44*†	*.72 ± .22*
B. Inflammatory (9)	*1.95 ± .91*	*.44 ± .27*
t, p (*vs* Group A)	.354, >.05	2.568, <.02
C. Rheumatoid (11)	*1.96 ± .53*	*.52 ± .24*
t, p (*vs* Group A)	.578, >.05	2.131, <.05
" (" " B)	.031, >.05	.688, >.05

† S.D.

¶ However, in(1) a thrombocyte sonicate was prepared, using entire thrombocyte sample drained and tube-wiped but unwashed to minimize prior thrombocyte damage. Aliquots of sonicate were then used for determinations, precluding any opportunity for washing thrombocytes prior to biuret determination. Plasma contamination was thus minimized but present; biuret determinations on dilute plasma samples revealed no significant difference in plasma protein levels between the 3 groups used. The nature of present study required early division of sample into aliquots while thrombocytes were intact and undamaged. This reduced sample volumes below that required for sonication. Since thrombocytes could not be sonicated, an aliquot of intact thrombocytes was available for biuret determination, and these thrombocytes were washed carefully as noted above. Overall amount of biuret positive material in thrombocyte preparations/100 ml of blood was thereby lessened although comparative relationship between Groups A and C of (1) and of the present study was essentially unchanged. The composition of Group B in present study differed from (1) in inclusion of more acute inflammatory states, so the A to B relationship cannot be compared between series.

TABLE III. Effect of Non-Autologous Plasma on Absorption by Thrombocytes of Serotonin Creatinine Sulfate.

Thrombocyte donor	mg thrombocyte protein /100 ml blood	mg thrombocyte protein/ recovery flask	Donor of 2 ml plasma /recovery flask	μg serotonin creatinine sulfate absorbed of 1 μg added
A (Noninflammatory)	25.6	1.71	A	.490
"		"	B	.392
B (Inflammatory)	63.5	2.14	B	.207
"		"	A	.164
C (Noninflammatory)	25.1	2.12	C	.688
"		"	D	.598
D (Inflammatory)	41.3	1.36	D	.146
E (Noninflammatory)	34.1	1.85	E	.682
"		"	F	.589
F (Rheumatoid)	57.7	2.11	F	.282

bocytes from patients with both non-rheumatoid and rheumatoid inflammatory states absorbed serotonin creatinine sulfate less completely than did thrombocytes from patients with no evidence of inflammation, although the added 5-HT/ml plasma was the same in all groups, number of thrombocytes/flask was essentially equal in all groups, and time interval for absorption was rigidly controlled. The decrease was probably significant ($p<.02$) in the inflammatory Group B and possibly significant ($p<.05$) in the rheumatoid Group C.

Table III shows the effect of non-autologous plasma on absorption by thrombocytes of added serotonin creatinine sulfate. Absorption of 5-HT by inflammatory thrombocytes showed the usual lesser value on comparison with noninflammatory thrombocytes when in autologous plasma. When plasmas were reversed so that homologous but not autologous plasma was added to thrombocytes, some depression of absorption of 5-HT was noted. However, the effect of inflammatory plasma on non-inflammatory thrombocytes and of noninflammatory plasma on inflammatory thrombocytes was essentially the same. The noninflammatory plasma did not improve the ability of inflammatory thrombocytes to absorb 5-HT. In presence of inflammatory plasma, noninflammatory thrombocytes were still able to absorb 5-HT in greater amounts than could the corresponding inflammatory thrombocytes in autologous plasma.

Summary. Under controlled *in vitro* conditions (involving equal numbers of human thrombocytes in autologous plasma to which was added an amount of serotonin creatinine sulfate that could be absorbed completely by a few normal specimens in the limited time period allowed), a possibly significant decrease in absorption of serotonin was noted with thrombocytes from patients with non-rheumatoid inflammatory states ($p<.02$) and with rheumatoid arthritis ($p<.05$), as compared to individuals with no demonstrable inflammation.

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Received May 15, 1959. P.S.E.B.M., 1959, v102.

Changes in Weight and Composition of Stomachs and Small Intestines of Rats after Vagotomy.* (25138)

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Recently we demonstrated that vagotomy resulted in decreased secretin but increased pancreozymin content of small intestine of the rat(1). Routine observations on stomachs revealed a substantial increase in weight of this organ in the vagotomized as compared to control animals. The present study was undertaken to confirm and extend this finding.

Methods. Female rats of Wistar strain were paired according to weight. A subdiaphragmatic bilateral vagectomy was performed on 1 member of each pair, a mock operation on the other. Vagotomized and control animals were maintained in individual cages on a paired feeding regimen for 7-63 days. Animals were fasted 12 hours before sacrifice. The rats were sacrificed in pairs, stomachs and intestines removed, and wet weight, dry weight, and weight of fat free residue of tissues determined. Weight of food residue was also noted. In 12 cases glandular and non-glandular stomachs were separated and weight measurements made for the 2 portions.

Results. Mean values for measurements are given in Table I.

Discussion. The results show that following vagotomy a marked increase in stomach tissue weight and a less dramatic reduction in intestinal tissue weight occurred. Changes in fat and water content, which in stomachs seem to have occurred in the glandular rather than muscular portion, are too small to account for changes in weight.

It is possible that mechanical distension due to retained food in some manner caused hypertrophy of the stomachs. However, in 2 vagotomized animals there was no evidence of food retention, yet the stomachs were enlarged.

It is obvious from the data that the observed weight changes in stomachs of vagotomized rats are not due to edema or fat deposition. Nitrogen determinations made on some of the fat free residues indicate that increase

TABLE I. Effect of Vagotomy on Weight and Composition of Stomachs and Small Intestines of Rats.

No. of pairs of rats		Vagotomized	Control	Mean difference V − C	P
35	*Mean wt of food residue in stomach after 12 hr fast	7.00	1.77	+5.24 ±1.30	<.001
35	Mean wt of rats	130.7	134.76	−4.0 ±3.4	Not sig.
35	" wet wt of stomachs	1.55	.95	+ .60 ± .12	<.001
24	" wt of oven dried stomachs	.34	.24	+ .10 ± .03	<.005
24	" wt of fat free residue	.30	.20	+ .09 ± .03	"
24	" % water in stomach tissue	79.9	77.5	+2.4 ± .6	<.001
24	" % fat *Idem*	11.4	16.2	−4.8 ±1.6	<.01
24	" Ratio, stomach wt:body wt	.02	.01	+ .01 ± .0031	<.005
12	Mean ratio non-glandular : glandular portion of stomach: Wet	.46	.40	+ .06 ± .03	Not sig.
	Dry	.44	.36	+ .07 ± .44	*Idem*
12	Mean % water: Glandular	78.2	75.7	+2.5 ± .4	<.001
	Non-glandular	78.8	77.0	+1.8 ±1.0	Not sig.
12	" % fat: Glandular	9.6	15.1	−5.5 ±1.9	<.02
	Non-glandular	9.1	10.7	−1.7 ±1.7	Not sig.
27	Dry wt of intestine	.92	1.06	− .14 ± .06	<.05
16	% fat in intestinal tissue	15.3	21.5	−6.2 ±3.4	Not sig.
8	% water *Idem*	89.4	86.9	+2.5 ±1.2	*Idem*

* All weights in g.

* This investigation supported by Grant from Nat. Inst. Health.

in stomach weight is due to protein rather than carbohydrate or minerals. The underlying mechanism is unknown.

Summary. Vagal denervation produced a marked increase in stomach weight and a less dramatic loss of intestinal weight. Changes in fat and water content of these structures could not account for the observed changes in weight.

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Received May 18, 1959. P.S.E.B.M., 1959. v102.

Action of Protamine on Production and Activity of Heparin-induced Clearing Factor.* (25139)

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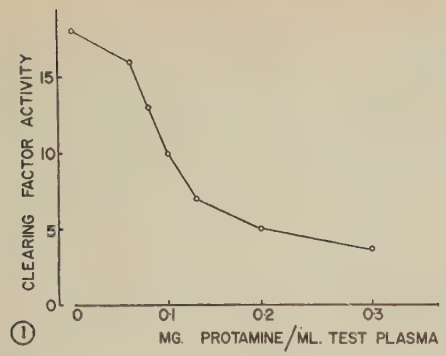
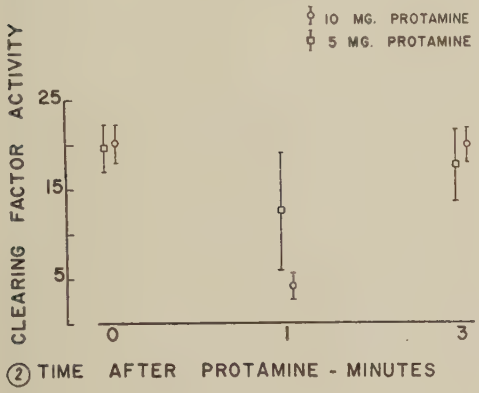
It is well established that protamine inhibits clearing factor *activity*. This inhibition can be demonstrated *in vivo*(1,2) and *in vitro*(3, 4). The effect of protamine injections on clearing factor *release* is not clearly established. Bragdon and Havel(5) showed that protamine caused hyperlipemia in fasting rats, and this has been confirmed by several other workers. Bragdon(6) subsequently found that protamine inhibits the disappearance of injected phospholipids from blood. He concluded that this was the result of anti-heparin activity of protamine. Similar conclusions could be drawn from the work of French and Morris(7) who injected C^{14}-labelled chylomicrons obtained from lymph of rats. They found that heparin caused an increase in rate of removal of chylomicron while protamine caused a decrease. According to the hypothesis of Robinson and French(8), clearing factor occurs normally on the capillary surface and may be displaced into the blood stream by injection of heparin or by adsorption on circulating chylomicra. Our recent results(9) support such an hypothesis. The question arises whether protamine may inhibit *release* of clearing factor by attaching itself to the capillary surface. Experiments were carried out to distinguish between an inhibitory effect on *release* of clearing factor and inhibition of clearing factor itself. Determinations were made first on the time protamine remained in the circulating blood after injection and, secondly how long after a protamine injection was it before a challenging dose of heparin would elicit full release of clearing factor.

Materials and methods. Protamine. Commercial product (Connaught Medical Labs) prepared as 1% solution, was used. *Clearing factor assay* was carried out by the method of Monkhouse and Mackneson(9). Lipemic substrate was prepared by adding either commercial fat emulsion, "Ediol," or chylomicrons to pooled oxalated dog plasma previously heated to 56°C for 5 minutes and stored at –20°C. Changes in turbidity were measured in Coleman Junior spectrophotometer at 500 mμ after 1 hour incubation at 37° C, and the activity expressed as changes in transmission for 0.1 ml of plasma being assayed. *Clearing factor release.* Each rat was given 50 units of heparin in 1 ml of saline solution intravenously, and the post heparin plasma taken 6 minutes thereafter. *Heparin* was kindly supplied by Connaught Medical Labs. Blood was removed from rats *via* exposed jugular vein. All samples were taken into one-tenth volume of 3.8% trisodium citrate solution. *Fat diet* contained approximately 40% protein (7% casein, 28% alcohol extract of peanut meat, 5% soya protein) 34% lard, 2% cholesterol, 10% sucrose and adequate vitamin supplements.

Results. Fig. 1 illustrates the relationship between protamine concentration and inhibition of clearing activity. Various dilutions of protamine were made with pooled citrated rat plasma. To each of 2 tubes, one containing

* This work was supported by grants from Ontario Heart Fn. and Defence Research Board of Canada.





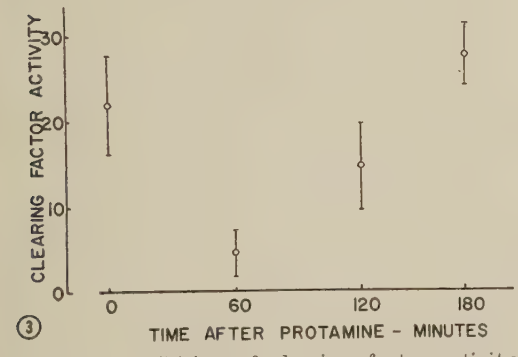


FIG. 1. Inhibition of clearing factor activity with different concentration of protamine added *in vitro*.

FIG. 2. *In vitro* clearing factor inhibitory activity of plasma withdrawn from rats at different times after they had received intrav. protamine. Each point is represented by 4 animals. Mean values and stand. dev. are shown.

FIG. 3. Inhibition of *release* of clearing factor in rats following intrav. inj. of 5 mg of protamine. Each point represented by 4 animals. Mean values and stand. dev. are shown.

0.05 ml of post heparin plasma and the other 0.10 ml was added 0.2 ml of various protamine dilutions. Each tube contained 1.6 ml of lipemic substrate and total volume was adjusted to 2.0 ml with saline. Increasing inhibition was obtained with plasma protamine concentrations up to 0.20 mg/ml plasma.

Protamine was injected intravenously into rats and samples of blood withdrawn every minute thereafter. Fig. 2 shows that protamine leaves the circulation extremely rapidly. Even with doses as high as 10 mg/rat no significant inhibition of clearing factor was observed with plasma obtained from animals 4 minutes after they received the protamine. When injections of protamine were made intraperitoneally only slight inhibition of clearing factor was obtained with plasma from injected animals. If inhibition did occur it was usually detectable in plasma between 30 and 60 minutes after injection.

In contrast to its transient stay in the circulation, a single injection of protamine has a prolonged effect on *release* of clearing factor. Fig. 3 shows the effect of intravenous injection of protamine on release of clearing factor activity by heparin. Twelve rats each received 5 mg of protamine intravenously. At each of 3 intervals, 60, 120 and 180 minutes thereafter a group of 4 rats received 50 units of heparin intravenously and 6 minutes later a sample of blood was withdrawn for clearing activity assay. There was still a decrease in ability of rats to release clearing factor 2 hours after protamine injection. This experiment was repeated with intraperitoneal injections of protamine in doses of 10 mg/rat. Again inhibition of clearing factor release was obtained, but to a much lesser degree and the results were more variable. It is worth noting that inhibition could be obtained in some animals as early as 5 minutes after injection.

Experiments were then carried out to determine whether repeated daily injections of protamine over 2 weeks would result in a more permanent impairment of clearing factor production. In a preliminary experiment intraperitoneal injections of protamine, saline or heparin were given. One ml of one of the solutions was given daily for 2 weeks to each animal. The protamine and heparin solutions both contained 10 mg/ml. Three of the rats receiving heparin died with intraperitoneal hemorrhage. Twenty-four hours after the last injection each animal received the stand-

ard 50 units of heparin and 6 minutes later a sample of blood was removed for clearing factor assay. The protamine treated animals showed no decrease in ability to produce clearing factor as compared to saline injected animals.

The experiment was repeated and extended to include variations in diet and temperature. Two groups of 24 rats each were used. One group was kept at room temperature and in a refrigerated room at 2°C. Animals were given a chow diet and maintained in their respective environments for 6 months. Six weeks prior to measuring clearing factor production, half of the rats in each group were put on a fat diet. After 4 weeks on the diet, half of the rats on the fat diet and half of those on the chow diet were given daily intracardial injections of 10 mg of protamine for 2 weeks. Twenty-four hours after last protamine injection, all animals were tested for ability to produce clearing factor. There was no significant decrease in ability to release clearing factor in the protamine treated rats when compared with controls. However, animals in the cold appeared to release more clearing factor than those at room temperature.

The effect of cold adaptation was repeated. Clearing factor release was compared in 10 rats which had been on a chow diet in the cold for 6 months with 10 rats of same age kept at room temperature for similar time. This time, clearing activity was measured according to rate of change in transmission/minute for the first 15 minutes incubation. This is considered to be a more sensitive test for enzyme activity(4). The results are shown in Table I. It can be seen that animals kept in the cold, release more clearing factor activity following standard heparin injection. Only one of 10 rats kept at room temperature had a value as high as animals in the cold.

Conclusions. (1) Samples of plasma taken from rats one to 3 minutes after they received an intravenous injection of protamine will inhibit clearing factor. Degree of inhibition varies with dose given, but even with doses as high as 10 mg no inhibition can be detected in the plasma 4 minutes after injection. An intravenous dose of 5 mg of protamine inhibited *release* of clearing factor for a period of more than 2 hours. It would seem that protamine which has been so quickly removed from the circulation, has been deposited at sites of clearing factor release. The simplest explanation, but by no means the only one, would be to assume that the site of clearing factor release is the endothelial lining of the vessels. This explanation would be in support of the hypothesis proposed by Robinson and French (8). (2) With intraperitoneal injections of protamine, inhibition of clearing factor release was detected in some animals within 5 minutes, whereas inhibitory activity was not detected in plasma until 30 to 60 minutes after injection, if at all. This was probably due to the fact that protamine was removed from the circulation almost as rapidly as it entered, and its removal blocked release of clearing factor. (3) Experiments in which protamine was injected daily for periods of 2 weeks indicated that the reported increase in atheromatous plagues(10) was probably not attributable to any prolonged impairment in clearing factor production. There would however be daily periods of from 2 to 4 hours immediately after each injection when there would be an impairment and this could be a contributing factor. (4) The increased ability to release clearing factor in animals adapted to cold environment may be associated with the general increase in metabolic rate. This increase in level of enzyme activity brought on by a physiological change encourages the thought that it has indeed a physiological function.

TABLE I. Clearing Factor Activity in Rats. Expressed as rate of change of transmission/min./ml of post heparin plasma. Figures are avg values and their stand. dev. based on estimates from 10 animals in each group. The differences are significant ($p = 0.005$).

Exposed to cold	At room temp.
14.7 ± 1.4	11.1 ± 2.2

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Received June 1, 1959. P.S.E.B.M., 1959, v102.

Differential Survival to Leukemia as a Function of Infantile Stimulation in DBA/2 Mice.* (25140)

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The influence of environmental factors on physiological activity has been well documented. The effects of emotional stress(1) and periodicity(2) are examples of environmental influences on physiological functioning. Recently there has appeared evidence to indicate that the nature of environment during organism's infancy exerts profound influence on physiological activity later in life. For example, rats which received extra stimulation during infancy show less mortality(3) following 120 hours of food and water deprivation and less adrenal hypertrophy 24 hours following an injection of hypertonic glucose(4) than do nonstimulated controls. This communication presents findings concerning the effects of infantile stimulation on length of survival following implantation of leukemia 1210 in DBA/2 mice.

Materials and methods. Ninety mice were used. Litters were assigned at birth alternately to either the stimulated or non-stimulated condition. Experimental treatment consisted of removing the pup from the nest, placing it in a 2 x 3 x 6″ compartment for 3 minutes, then returning the infant to the nest. This procedure was initiated on day following birth and was continued once daily until weaning on day 24. There were 42 mice in this treatment group. The non-treated subjects (N = 48) remained in the nest and were not treated in any manner until weaning. On day 24 all mice were weaned and placed in group cages. No animals were manipulated from weaning until time of implantation. All animals were implanted interperitoneally with 2 x 10^6 leukemic cells in 0.1 ml volume of Locke's solution when they reached a weight of at least 18 g, *i.e.,* between ages 45 to 55 days. The tumor used, L-1210, is a transplantable lymphoid leukemia induced in DBA/2 mice(5), maintained in the tumor bank at Battelle Memorial Inst. by weekly implantation of 2 x 10^6 tumor cells into the peritoneal cavity. This tumor has been very consistent in causing death in 100% of implanted animals with mean survival time over the past year between 8 to 9 days, and with a latent period of 2 to 3 days after implantation.

Results. (Table I) shows that animals stimulated by manipulation during postnatal period have lower survival times than non-stimulated mice. In the first and second replications there was no overlap between groups, thus obviating the need for statistical analysis. A median test was used for the third replication and yielded an X^2 of 20.16 significant beyond the .001 level of probability. Although there were some differences between the replications, these may in part be attributable to the fact that a transplantable tumor shows changes during the course of repeated transplantation. These changes may manifest themselves as slight variations in mean survival time of animals implanted. Furthermore, the experimental subjects, although highly inbred, may show fluctuation in re-

* This investigation was supported by research grant from Inst. of Mental Health, N.I.H., P.H.S.

TABLE I. Survival Time in Days after Tumor Implantation.

		N	5	6	7	8	9	10
Rep 1	S	10			10			
	NS	12					12	
2	S	7	7					
	NS	8					8	
3	S	25		18	6	1		
	NS	28		3	23		1	1

S = Stimulated during infancy.
NS = Nonstimulated controls.

sponse to implanted tumors because of seasonal variation, or the state of the colony in relation to endemic disease. However, the direction of differences is consistent and significant between replications.

In every test the early stimulated mice have shown significantly earlier death to the implanted leukemia.†

Discussion. These data are contrary to what might have been expected on the basis of previous research. Whereas in the past, viability has been increased by infantile stimulation, the opposite is found in these experiments.

It has been proposed that stimulation as used in this study, constitutes a stress for the infant organim. The effects of experience to chronic stress in infancy is hypothesized to modify the "neural stats"(6) resulting in a modified pattern of response to later stressful stimuli. More specifically this hypothesis states that the nature of the modification is a reduction of neurohumoral response to physiological insult. In view of certain constitutional factors such as size, developmental rate and genetic hyperemotionality of the DBA/2 mouse it appears that handling may be even more stressful to the mouse, thus reducing still further the neurohumoral response to later stress.

It is possible, therefore, that survival to the implanted leukemia may depend upon the organism's ability to produce a maximal neurohumoral response and when this response is effectively reduced less viability results.

These hypotheses are at best tentative. Any attempt to elucidate mechanisms must await further research. It has been suggested, however, that our results could be explained on the basis of a mild, inapparent infection caused by the handling procedure. This suggestion does not appear tenable for 2 reasons: (1) The background data for this study was the response of more than 3,000 mice implanted with L-1210. Many of these mice were also used in a study of prevalence of salmonella organisms in experimental mouse stocks. Survival time from leukemia was quite consistent and was not affected by presence of a low grade infection; and (2) the effects of infantile stimulation have been shown when the stimulating procedure did not involve handling(7).

Summary. DBA/2 mice were stimulated by being removed from the nest for 3 min. daily from birth to day 24. Control mice received no stimulation. Between days 45 and 55 all animals were implanted with leukemia 1210. The stimulated mice showed significantly shorter survival time than their nonstimulated controls. This effect is assumed to be a result of early chronic stress leading to a reduced neurohumoral response to later challenge.

† Since writing this report, 2 subsequent experiments have been done. One experiment using hybrid BDF/1 showed the same results as those presented here, and an additional replication using DBA/2 mice yielded the same significant results.

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Received June 1, 1959. P.S.E.B.M., 1959, v102.

Direct Measurement of Conduction Velocity in *In situ* Specialized Conducting System of Mammalian Heart.* (25141)

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Earlier attempts(1,2) to determine conduction velocity in the *in situ* specialized conducting system were made on excised hearts perfused with saline solutions: In the first case the value obtained was 0.75 m/sec, in the second 2-3.5 m/sec. Measurements made *in vitro* on isolated bundles of Purkinje fibers(3, 4) have been restricted to a single portion of the conducting system, the so-called false tendons. By using a pump-oxygenator which supplies both a total body perfusion and a Langendorf perfusion of the heart we found it possible to expose any part of the specialized conducting system and to record electrical activity under direct vision(5).

Methods. In these experiments large mongrel dogs were anesthetized with pentobarbital. After total cardiopulmonary by-pass the right atrium and right and left ventricles were opened by incisions in their free walls. The bundle of His, right and left bundle branches, free-running Purkinje fibers and their distal insertions were located by known anatomical landmarks and on the basis of local bipolar electrograms recorded through a freely movable exploring electrode. Small plastic electrodes containing from 3 to 16 fine silver contacts were then attached at desired locations by fine silk sutures which passed through the endocardium 2-4 mm from the recording site. Bipolar electrograms recorded simultaneously from fixed and exploring electrodes were displayed on an 8 beam oscilloscope and photographed on paper moving at 200 mm/sec. Temperature of endocardium at each recording site was determined from time to time by a small thermistor. In some experiments additional electrodes were attached to the atrium and ventricle for electrical stimulation. Distances between recording sites were measured directly at end of each experiment.

Results. Identification of local electrograms as records of electrical activity of the specialized conducting system rests upon both anatomical and physiological considerations. Action potentials of the His bundle and other portions of the conducting system are recorded from appropriate anatomical locations where well-defined tracts of the conducting system are known to lie. Clear-cut action potentials with rapid deflections and short durations are noted (Figs. 1 and 2) and it can be seen that these action potentials follow atrial activity and precede ventricular activity. When the vagus nerve is stimulated (Fig. 1 b) the interval between atrial activity and the action potential of the His bundle is greatly prolonged but no change is noted in the interval between activity in the bundle of His and the peripheral Purkinje fibers or ventricular myocardium. Also, during retrograde excitation (Fig. 1 d) the sequence of action potentials recorded from ventricle, peripheral Purkinje fibers, bundle of His and atrium shows the appropriate change.

The records shown in Fig. 2 permit determination of conduction velocity from the bundle of His to emergence of right and left bundle branches and from these sites to points, in the left ventricle, at which the anterior false tendon emerges from the septum and enters the papillary muscle and, in the right ventricle, to junction of Purkinje fibers with the anterior papillary muscle and free ventricular wall. Intervals and distances are listed in Table I. In this experiment, corresponding to records of Fig. 2, at heart temperature of 36°C, the conduction velocity from the bundle of His to left and right bundle branches is 1.2 - 1.3 m/sec. In contrast, conduction velocity in the free-running false tendons of left ventricle is 4.0 - 4.1 m/sec and in the right

* This work supported in part by grant from U.S.P.H.S. (H-3916).

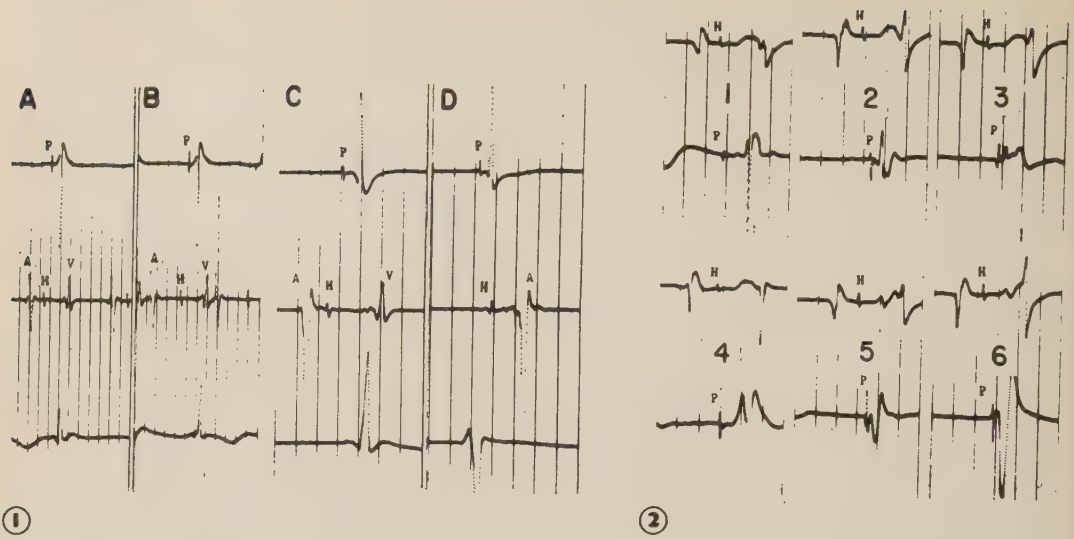

FIG. 1. Bipolar electrograms recorded from left interventricular septum (top trace), bundle of His (middle trace) and left anterior papillary muscle. P = Purkinje fiber electrogram; H = His bundle electrogram; A = Atrial electrogram; V = Ventricular electrogram. Records in A obtained prior to and in B during stimulation of left vagus nerve. Paper speed 100 mm/sec., time lines = 100 msec. C and D bipolar electrograms from same locations as in A and B recorded from a different heart. Records in C obtained normal sinus rhythm and in D during ventricular driving. Paper speed 200 mm/sec., time lines = 100 msec.

FIG. 2. Bipolar electrograms recorded from bundle of His (top trace) and peripheral Purkinje fibers at the following locations in one heart: 1—Right bundle branch; 2—junction of moderator band with right anterior papillary muscle; 3—junction of right false tendon with free ventricular wall; 4—left bundle branch; 5—emergence of left anterior false tendon from septum; 6—junction of anterior false tendon with left anterior papillary muscle. Changes in configuration of the ventricular complex on top trace are caused largely by changes in position of heart. Paper speed 200 mm/sec., time lines = 100 msec.

ventricle 4 m/sec. Average conduction velocity from the bundle of His to termination of the free-running Purkinje system is 2.45 m/sec for the right ventricle and 2.6 m/sec for the left ventricle. Similar values for conduction velocity in the bundle of His and free-running Purkinje fibers have been obtained in other experiments. In the former the range is from 1 to 1.5 m/sec and in the latter from 3 to 4 m/sec. When regular driving stimuli are applied to the ventricle at rates from 60-150/minute conduction velocity within the Purkinje system and bundle of His in the retrograde direction is the same as during normal activation. The maximum value found by Maeno(2) for conduction velocity in the *in situ* false tendons of the excised, perfused dog heart is of the same order of magnitude as our results.

TABLE I. Conduction Velocity in Specialized Fibers.

Location	Conduction time, msec.	Conduction distance, mm	Velocity, m/sec.
His to lt. bundle branch	7.5	9	1.2
His to rt. bundle branch	10	13	1.3
Lt. ant. false tendon	2.25	9	4.0
Lt. post. false tendon	7	29	4.1
Rt. false tendon	3	12	4.0

Summary. By use of a pump-oxygenator and cardiopulmonary bypass, bipolar electrograms have been recorded under direct vision from the bundle of His, the right and left bundle branches and peripheral Purkinje system of dog hearts. Conduction velocity from the bundle of His to bundle branches is 1.0-1.5 m/sec, while in the false tendons this value is 3-4 m/sec at 36°C.

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Received June 3, 1959. P.S.E.B.M., 1959, v102.

Variations in Post-Weaning Development of Ruminal Mucosa in Lambs.* (25142)

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Earlier workers(1-5) have suggested that diet has some effect on rumen development. This was not convincingly shown however, until reports of Warner and coworkers(6-8), which strongly indicated that chemical factors, probably in the form of active microbial fermentation products, act as stimulants for development of the ruminal papillae. The literature, however, appears devoid of reference to either qualitative or quantitative relationship of development of ruminal mucosa to post-weaning growth patterns in the animal. The stimulus for the present study developed from observations of rumina of sheep slaughtered at termination of an earlier feeding trial. Some lambs in the earlier trial had gained poorly, and a striking relationship existed between gain and ruminal development although all lambs were of an age and were receiving a diet which was expected to have conditioned full development of the rumen. The sheep which gained weight slowly had poor development of the rumen while more rapidly gaining ones had rumina which had thickened mucosae with well-developed papillae. As far as we were aware, such a relationship between structural development of a tissue and rate of gain of the animal had not been previously reported.

Procedure. Detailed observations were made on rumina from 42 feed-lot lambs upon slaughter at the end of a 114-day feeding period. The lambs weighed an average of 62.1 lb (range of 49-90 lb) at the beginning of the feeding trial, prior to which they had been maintained on a diet of mixed forages. Weight and gain data were available on all animals. The self-fed basal diet consisted of 25% oat straw, 24% beet pulp, 14% wheat, 16% oats, 18% linseed meal, and 4% sucrose. Stabilized Vit. A (Chas. Pfizer & Co., Inc.) was supplemented at the rate of 650,000 I.U. per ton of feed. Other than addition of 48 lb of K_2HPO_4 per ton of feed (added in an attempt to induce urinary calculi formation), the diet may be considered adequate. The sheep were fed in 5 groups, their diets differing only in antibiotic content (Table I). The antibiotics chlortetracycline and oxytetracycline were supplied as crude supplements (Aurofac and TM-10, respectively). As soon as possible after evisceration, rumen and reticulum were tied off, slit at the dorsal midline, emptied, and washed. Weights of the emptied whole ruminoreticulum were taken and gross observations made of color, length, and density of papillae of the various rumina. Sections of tissue were immediately removed from the anterioventral sac of the rumen and placed in 10% formalin. Formalin fixed sections from 28 of the animals were later separated into 2 sections: a "mucosal" layer, consisting of the epithelial lining, submucosa, muscularis mu-

* This investigation supported in part by grants from Amer. Cyanamid Co., Pearl River, N. Y. and Chas. Pfizer and Co., Terre Haute, Ind. The investigation is also a part of S-10 Beef Cattle Breeding Project which is cooperative between Southern State Experiment Stations and U. S. Dept. of Agric.

† Appreciation is expressed to Mr. Joe D. Robbins and Dr. T. D. Watkins, Jr. for their interest and assistance in the feeding experiment.

TABLE I. Effect of Antibiotic Supplementation on Gain and Rumen Characteristics.

Group	I			II			III			IV			V		
Antibiotic	None			CTC,* 10 mg/lb feed			CTC,* 20 mg/lb feed			None			OTC,* 10 mg/lb feed		
No. of lambs	8			8			10			8			8		
Avg initial wt, lb	59	± 17	†	61	± 17		64	± 19		64	± 18		64	± 19	
" daily gain, lb (114 days)	.35	± .02		.41	± .02		.43	± .10		.38	± .10		.37	± .05	
Idem (last 28 days)	.24	± .15		.25	± .09		.40	± .10‡		.21	± .20		.29	± .14	
Avg reticulorumen wt, g	773	±166		843	±153		839	±163		734	±153		818	±132	
" length papillae, cm	5.3	± .33		5.3	± .38		5.7	± .29		4.7	± .21		4.7	± .35	
" width index, papillae‖	6.8	± 1.9		6.0	1.1		6.5	± 1.2		6.5	± 1.4		6.6	± 1.5	
" density index, papillae‖	6.4	± 1.1		7.5	.5		7.9	± .3		7.4	± 1.3		7.5	± 1.3	
" color index, mucosa‖	5.7	± 1.1		5.7	.9		7.4	± .5 §		6.3	± 1.4		7.0	± 1.9	

* CTC = Chlortetracycline, OTC = Oxytetracycline. † Stand. dev. ‡ Significantly different from Groups I & IV at .05 level of probability.
§ Significantly different from Groups I & IV at .01 level of probability. ‖ Indexes based on arbitrary classification of 1 to 9 with increasing avg relative width, density and color. See text.

cosa, and mucosa proper, and a muscle layer consisting of the striated muscles of the rumen wall. The sections were air-dried and weighed. From these weights the percent mucosa was calculated. Mucosal percentages ranged from 51% to 75%. Much of the tabulated data were determined from photographs of the anterioventral sac. Color index, relative papillary density, and relative papillary width of each rumen were estimated by placing these photographs into 9 classes of color, density, or width gradations established upon study of the variations observed in the rumina of over 70 lambs. Each class was assessed a value of 1 through 9 in order of increasing color, density, or width and these values were used as the basis for estimation of the relationships. Fig. 1 shows approximate range in color in the present experiment and Fig. 2 presents variation in density. Sections of formalin fixed tissues were taken approximately 2 inches immediately cranial to the anterior pillar, embedded in paraffin, sectioned, and observed histologically. Papillae lengths also were estimated from these paraffin blocks and varied from 2.5 to 7.5 mm. The anterioventral sac was the focal area of study, not only as a convenient point of reference between our data and those in the literature, but also because it appeared that variations in development in this area reflected variations in the rumen as a whole more so than any other segment. The 114-day gains used in calculating relationships were those made during the entire feeding period. The terminal 28-day gains were evaluated in an effort to estimate the relationship of the more immediate gain to the various characteristics of the rumen.

Results. The data showing the effect of antibiotics on 114 day gains, terminal 28 day gains and mucosal characteristics are summarized in Table I. Such differences in the 114 day gains as may have been elicited by the antibiotic treatment were not significant statistically. The lambs receiving 20 mg of chlorotetracycline per lb of feed showed a significantly higher average gain ($p<0.05$) during the terminal 28 days of the experiment.

Weights of total tissue of the ruminoreticulum and relative papillary densities were

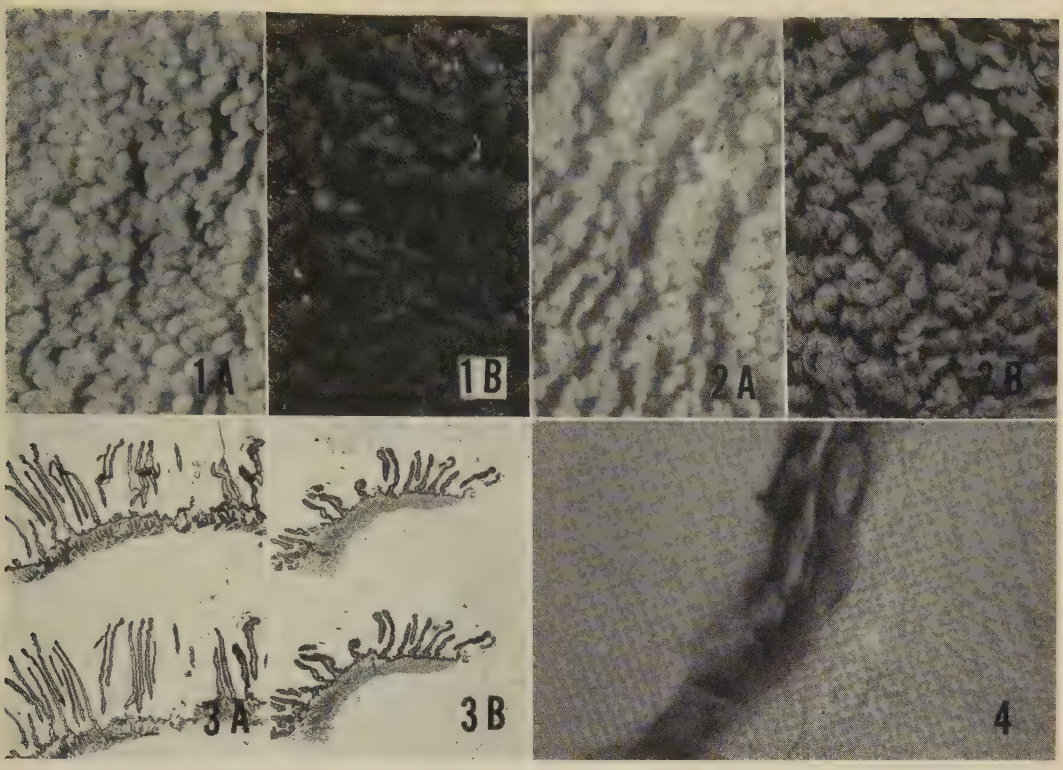

FIG. 1. Photograph of floor of anterioventral sac of rumen showing wide variation in color noted between individual lambs. A was assessed relative value of 4; B, value 9, maximum.

FIG. 2. Example of variation noted in density of papillae between individuals. A was assessed the relative value of 3 while B was classed as 9, a maximum density.

FIG. 3. Cross section of rumen wall showing variations in length between lambs. Papillae in A measured 7 mm and in B, 4 mm in length.

FIG. 4. Formalin fixed, frozen blade-sectioned, unstained rumen epithelium showing brownish-black keratinized layer. 970 ×.

slightly increased by the antibiotics but the differences were not statistically significant. Mucosal color, though not significantly affected by low levels of either antibiotic, was significantly intensified by 20 mg chlorotetracycline per lb of feed.

Coefficients of correlation were calculated to evaluate overall relationships between gains and various ruminal characteristics (Table II). A number of these coefficients were of sufficient magnitude and statistical significance to verify the working hypothesis that

TABLE II. Coefficients of Correlations among Gains, Initial and Final Weights, and Mucosal Characters.

	Gain, last 28 days	Initial wt	Final wt	Reticulorumen wt	% mucosa	Papillary length	Papillary width	Papillary density	Color index
Gain, 114 days	.51†	.34*	.67†	.60†	.21	.59†	.09	.31*	.12
" , last 28 days		.07	.27	.65†	.54†	.36*	.30	.40†	.39*
Initial wt			.93†	.64†	−.14	.20	−.15	.34*	.13
Final "				.74†	−.05	.40†	−.08	.39*	.12
Reticulorumen wt					−.06	.46†	.19	.48†	.20
% mucosa						.12	.30	.10	.25
Papillary length							.18	.32*	.04
" width								−.08	−.04
" density									.56†

* Significant at .05 level of probability. † Significant at .01 level of probability.

ruminal development is in part related to rate of gain of the animal. In addition, analyses of covariance within groups indicated that, with exception of the relation of mucosal color to terminal 28-day gains, statistically important relationships were not significantly affected nor explained by the effects of antibiotic treatment. The indication is that rumen development was largely an individual characteristic of physiologic consequence.

The highly significant correlation of total weight of ruminoreticulum to average daily gain (Table II) was as might be expected from the wide range of final body weights (69-150 lb). The weight of total ruminoreticulum tissue was largely proportional to overall size of the animal and feed-lot gain was also a determinative part of that size. Enhanced rates of gain, however, could have been due also to a number of factors including an increased functional capacity of the larger rumen.

Specific indications of a functional relationship between ruminoreticulum development and animal gain are noted upon closer inspection of the coefficients of correlation between total organ weight, final body weight, and terminating 28-day gain instead of total 114-day gain. Although correlation of the 114-day feed-lot gain to weight of the ruminoreticulum can be largely accounted for by the significant parallelism of both variables with final body weight, a similar conclusion cannot be stated for the relationship of ruminal tissue weights to more immediate changes in weight of the animal. The coefficient of correlation of terminal rates of gain to final weights was only 0.27. Significantly more of the variance in ruminoreticulum weights can be accounted for by the estimate of multiple correlation of tissue weight with terminal rate of gain and body weight ($R = 0.88$; $R^2 = 0.77$) than with either terminal gain ($r^2 = 0.42$) or body weight ($r^2 = 0.55$) alone.

Percentage of mucosa, which may represent functional development of the rumen(8), was likewise significantly correlated with gain during the terminal 28 days but to a smaller extent to gain during the entire period. Mucosal development, however, may not be independent of development of underlying muscle.

Histological evaluation of sections of the anterioventral sac suggested that papillary length is a definitive criterion for evaluating mucosal development (Fig. 3). The relationship of papillary length to rate of gain appears important but, in this instance, lengths of the papillae seemed to be more closely related to long-term rates of growth than to the more immediate gains. Only a relatively small part of the variance in length of papillae could be explained on the basis of rumen weight ($r^2 = 0.209$), initial weight ($r^2 = 0.042$), or final weight ($r^2 = 0.156$). Thus partial coefficients of correlation between 114-day gain and papillary length independent of rumen weight ($r_{gp.r} = 0.437$), independent of initial weight ($r_{gp.iw} = 0.560$), and independent of final weight ($r_{gp.fw} = 0.470$) were all highly significant statistically.

Papillary density, like papillary length, serves as an evaluation of surface area available for absorption or other functions that the rumen may perform. Though not so highly related as was length, estimated density was correlated significantly with gains in both periods. The multiple coefficient of correlation of 114-day gain with relative length of papillae and relative papillary density ($R = 0.60$), however, was only slightly greater than that with papillary length alone.

Widths of papillae were examined because of their great variation among different individuals, but although the relationships to gains were positive, they were small and not significant statistically.

The unexpected result of these studies was the extent to which mucosal color of the rumen was related to gain. The correlation was not high but was of statistical significance for the last 28 days of trial. Attempts were made to localize this color and apparently it is restricted to the cornified layer. Fig. 4 shows a section of formalin fixed, frozen blade-sectioned and unstained rumen epithelium. Where no organic solvents were used in the histological preparations, the cornified layer retained a dark brownish color which was like the coloration noted in gross examination of the rumen. An implication with respect to color is that it may represent products of microbial activity especially since the color appears to be affected by dietary antibiotics.

If so, color may provide additional means of evaluating bacterial action and thereby be also a means of evaluating the influence of dietary factors on rumen flora.

The coefficients of correlation obtained in this study are not high, but it should be noted that they are based on relatively unrefined descriptions of the rumen. From the data presented, however, it appears that variations in development of the ruminoreticulum in general and of the rumen mucosa specifically are a structural expression of rate of growth, and possibly of capacity for growth as it is affected by general nutritional state, inherent growth impulse of the animal, or capacity of individual sheep to adapt to a functional symbiosis.

Summary. Rumina of 42 sheep, fed an adequate diet, were examined for assumed characteristics of development: total weight of reticulorumen, percent mucosa, and papillary length, width, and density. The significant correlations of rumen weight, papillary length and papillary density to rate of gain present strong supportive evidence that growth and development of the animal as a whole is related to and may be dependent upon ruminal development. Evidence is presented indicating that dietary antibiotics, chlortetracycline and oxytetracycline, may have a slight effect on mucosal development. An unexplained relationship of mucosal color and gain is presented.

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Received June 4, 1959. P.S.E.B.M., 1959, v102.

Unstable Inhibitory Mechanism in Rheumatoid Sera.* (25143)

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Serial latex fixation tests in a large group of patients with rheumatic diseases revealed a pattern of agglutination with only the euglobulin fraction of serum and not with the whole serum. Repeat freezing and thawing of whole serum in the course of these studies appeared to convert a hitherto negative whole serum latex agglutination to a positive latex whole serum agglutination. These experiences suggested an active inhibitory mechanism that might be relatively unstable.

Materials and methods. The patients were from the outpatient and inpatient services of the Maimonides Hospital. The Fraction II latex particle tests were performed by the method of Singer and Plotz(1). Squibb's lyophilized fraction II was used throughout. Euglobulin fractionation was performed using Ziff's method(2) but modified so that the precipitate was redissolved in glycine saline buffer in order to utilize the latex system. Sheep cell agglutinations were also performed by the method of Ziff and the serum, as is customary, was inactivated by heating to 56° for ½ hour before absorption. Standing sera latex particle agglutinations were performed as follows: Immediately after being separated from whole blood, serum was allowed to stand for 48 hours at room temperature. Latex particle agglutination was then carried out in the usual manner. In other procedures, 2-fold and 4-fold additions of normal fresh serum from the same patient or from healthy donors were

* Supported by grants from the N. Y. Chapter of Arthritis & Rheumatism Foundation and from the Charles Pfizer Co.

TABLE I. Comparison of the Euglobin Latex, Standing Latex and Chemical State in 9 Patients.

Diagnosis Stage	Grade	Whole serum	SCA	Euglobin latex	Standing latex	Nodules	No. of previous neg. whole sera	Eu. + tests
2	3	Neg.	Neg.	1:224	1:320	0	2	
2	2	"	"	1:112	"	+	3	On 4th t. W.S. +
1	1	"	"	1:224	1:160	0	0	
1	2	"	"	1:56	"	+	1	
R. F.		"	"	1:224	1:80		0	
1	2	"	"	1:112	"	0	0	
? R. A.		"	"	1:28	Neg.	+	3	
3	2	"	"	1:112	"	0	2	3
? R. A.		"	"	1:56	"	0	3	

added to these standing sera and latex particle agglutination again performed.

Results. Nine sera (7 definite rheumatoid, 2 questionable rheumatoid) that had previously demonstrated a positive euglobulin latex agglutination and negative whole serum latex agglutination were studied. Allowing these sera to stand for 48 hours and then repeating the serological test on whole serum produced agglutination in the latex system in 6 sera (all definite rheumatoid) where previously there had been none. The titer of agglutination was similar in magnitude to that previously demonstrated with euglobulin if dilution differences are taken into consideration (Table I). Many normal sera from healthy volunteers were repeatedly tested in the same way and in each instance were negative.

In each of the 6 instances, the patients' own fresh serum and pooled normal human sera were added in 2-fold or 4-fold dilutions to the standing sera and the tests then repeated. However, in no instance did agglutination not occur.

Sheep cell agglutinations on the above sera were all negative.

Discussion. Previously, Ziff *et al.*,(3) and Bayles' group(4) have adequately demonstrated that by doing routine euglobulin dialysis one may add sensitivity to the serological test in rheumatoid arthritis whether one uses sensitized sheep cells or latex particles. In addition, Ziff demonstrated, as had Olsen and Rantz(5) that inhibitor could not be completely removed by euglobulin dialysis. Clark's group has suggested that complement 4′, a relatively heat stabile component of serum, was an inhibitor(6). However, heating to 56° for ½ hour should remove this factor as was done in the sheep cell test, but these tests on the same group of patients were negative. In addition, by a series of involved biochemical steps Singer(7) has suggested that complement is of no importance in performing the latex particle agglutination test.

Our studies confirm those of Olsen and Rantz and Ziff in that we have adequately demonstrated that inhibition exists clinically and serologically, the latter apart from the euglobulin fraction.

Gerber, working in Ragen's laboratory (personal communication), suggested that the alpha II fraction of serum may be an inhibitor. We cannot say at this stage in our study whether our inhibitor is an alpha II fraction, or whether this is inhibition as a result of precipitation at room temperature of gamma globulin with rheumatoid factor. Since we cannot restore inhibition, the possibility that there is an involved physico-chemical relationship, which is more of an inhibiting mechanism, rather than an active inhibitor, exists and must be studied.

The foregoing studies also suggest that an inexpensive substitute for euglobulin dialysis may be to allow the serum to stand before proceeding to dialysis. Indeed, further studies may suggest that serological tests be done only on standing sera.

Summary. Serological studies have suggested an unstable inhibitor mechanism that can be unmasked by exposure of serum to room temperature. This may be the responsible mechanism for the pattern of positive euglobulin agglutination and whole serum

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Received June 12, 1959. P.S.E.B.M., 1959, v102.

Studies of Properdin System in Human Cancer Cell Cultures.* (25144)

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The demonstration of low serum properdin titers in many patients with advanced neoplastic diseases(1-5) and the correlation of this phenomenon with diminished ability to reject homotransplants of cultivated human cancer cells(1,6) impels direct study of the effects of properdin on growth of neoplasms. The present paper reports failure of properdin to affect growth of human cancer cells in tissue culture, and the effects of incubation in tissue cultures on complement components and properdin.

Methods. Properdin (P) was prepared from normal human serum by 2 methods: adsorption onto zymosan (Z) at 17°C and subsequent elution of P from resulting P-Z complex(10), or a modification of the Cohn serum fractionation technics utilizing ethanol precipitation(16). These preparations were supplied by Dr. Louis Pillemer of Western Reserve University and Dr. Benjamin Sanders of Merck, Sharp and Dohme Labs respectively. Human serum reagents lacking P (RP) or lacking P and C'3 (R3) but containing all other components of the properdin system were prepared by adsorption with zymosan at 17°C and 37°C respectively(9,10). In studies of P prepared by Z adsorption, the same serum was the source of both P and RP. All preparations of P, RP, and R3 were tested in tissue cultures singly (*i.e.* in absence of complete P system) to eliminate preparations with innate cytoxicity. They were stored at –10°C or –60°C and thawed at room temperature immediately before use. P analyses were performed by the zymosan (hemolytic) technic of Pillemer without modification(10). All RP, R3, embryo extract, pleural fluid, and ascitic fluid preparations used had no titratable P (<1. U/ml). Parallel C'3 titrations on RP preparations in presence and absence of Z suggest that residual P was less than 0.5 U/ml. Since the tissue-culture media contained not more than 50% serum products, it follows that media without added P had residual P levels of <0.5 U/ml and probably <0.25 U/ml. For convenience these media are referred to as properdin-free. C' and C' components were assayed by the technic of Ecker, Pillemer and Seifter(17) except that 50% hemolysis end point was used instead of 100%. The hydrazine method was used for preparation of the R4. Percent hemolysis was estimated by visual comparison with standards prepared from same erythrocyte suspension used for the test. All P assays and all C' and C' component assays from a single experiment were done simultaneously using the same reagents throughout. At least one normal human serum standard (and in properdin studies, a purified properdin standard) were included in all titrations to check

* These studies supported by research grants from Nat. Cancer Inst., U.S.P.H.S., and Phoebe Waterman Fund. The authors are also greatly indebted to the late Dr. Louis Pillemer and his staff, without whose cooperation and assistance these studies could not have been initiated.

the reagent systems. All specimens were stored in mechanical deep freeze (–60° to 70°C) from time of collection to time of titration. Two human cancer cell lines, Toolan's HEp #2(7) and Osgood's J-111(8), were cultivated for approximately 12 months prior to these experiments in properdin-free tissue culture media consisting of 45% Gey's salt solution, 5% chick embryo or beef embryo extract, and 50% ascitic or thoracic fluids from cancer patients. Subcultures were then planted in tubes and fed with 1 ml of another properdin-free medium consisting of 45% Gey's or Eagle's solution, 5% chick embryo extract, and 50% properdin-free serum (RP or a mixture of RP and R3). Gey's solution (11) was used for HEp #2 cells, and Eagle's solution(12) for J-111 cells. After cultivation in these media for 1-3 weeks to allow adaptation of cells to the RP serum, P (in solution in Gey's or Eagle's solution) was added to some to produce the desired final concentrations of P. Cultures were incubated at 37°C in stationary tubes without plasma clot and were refed with the appropriate media at intervals of 1 to 7 days. Representative samples of the old media which were removed when refeeding were titered for residual properdin and complement. Maximum pH variation in the cultures was 6.5 to 7.5. Magnesium and calcium ion concentration in the various media were calculated to be in the magnitude of 1×10^{-3} and 2×10^{-3} molar respectively. In studies of homotransplantability (Exp. 1), cultures were refed every 4-7 days, and after observation for 3 weeks or more, tubes containing identical nutrients were trypsinized, pooled, and transferred to milk dilution bottles for further cultivation in 10 ml of same media. Finally, they were harvested by trypsinization, washed twice, resuspended in Gey's salt solution, counted by hemocytometer, and transplanted subcutaneously to volunteer patients with advanced cancer and low serum properdin titers. Implant sites were observed daily for evidences of growth of homotransplanted cells and after nodules appeared they were measured daily until excised simultaneously for comparative microscopic study. In studies involving serial titration of C′ and P (Exp. 3), cultures and blanks were in milk-dilution bottles containing 12 ml of medium. For this study the medium was 35% RP, 15% R3, 5% chick embryo extract, and 50% Gey's solution or properdin in Gey's solution. Aliquots of 2 ml were removed from each bottle (and not replenished) at each of the 4 time intervals.

Results. Exp. 1. Prolonged exposure to properdin followed by homotransplantation. HEp 2 and J-111 cells were cultivated in media containing 0, 5, or 20 U of P/ml for 6 weeks with replenishment of medium every 5 days. There was no effect on cell morphology or multiplication rate as judged by microscopic observation of stained and unstained cultures. Total cell counts at end of cultivation period indicated that cells had increased between 6-fold and 30-fold without relation to P content of the medium. These differences are not significant. After each refeeding the cultures contained a complete complement system as measured by hemolytic effect of the medium on sensitized sheep erythrocytes, but complement activity did not persist much longer than 24 hours (see Exp. 3). Thus, these cells were only intermittently exposed to a complete P′ system. P levels in these cultures fell 50% or less during 5 days of incubation. Homotransplantation of these HEp #2 cells into a volunteer patient with advanced malignant melanoma and serum P level of <1 U/ml resulted in growth of a nodule at each implant site, with no significant differences in growth rate or final nodule diameters. Biopsies of all 4 nodules on day 14 showed identical histologic appearance with good growth of the implanted cells and no inflammatory or other regressive changes. Homotransplantation of the J-111 cells into another melanoma patient with a serum properdin titer of <1 U/ml produced only a vaguely palpable thickening at implantation sites by the 27th day after inoculation when the patient died. At autopsy the only J-111 nodule which could be located for excision was that produced by cells which had been grown in a properdin concentration of 20 U/ml. In histologic sections there were sheets of healthy appearing cells of the implanted type and no inflammatory or regressive change. Although this reimplantation

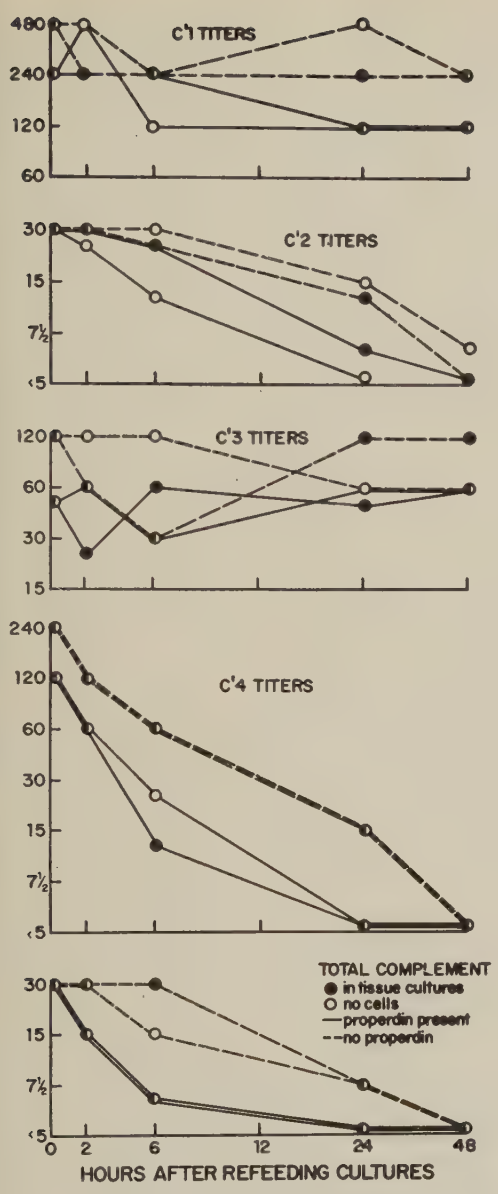

FIG. 1. Total complement and complement component titers in tissue culture media in Exp. 3 (see text). Black dots (●) indicate media from HEp 2 cell tissue cultures. Open circles (○) indicate media from "blank" culture bottles, *i.e.*, no cells present. Solid lines indicate media containing properdin (20 U/ml without change for duration of experiment). Dotted lines indicate properdin-free media. Titers and rate of fall of C'1, C'2, C'4, and total complement were not significantly affected by cells, but in presence of properdin (with or without cells) titers were lower. Titers of C'3 varied erratically during first 6 hr in presence of properdin or cells but no significant changes persisted after 24 hr.

study of J-111 cells did not permit adequate comparison between the 4 preparations, it is clear that cell growth potential was not abolished by cultivation in the presence of properdin. In other cancer patients homotransplantation studies with J-111 cells grown in properdin-free media gave similar nodule growth and microscopic appearance.

Exp. 2. Cultures continuously exposed to a complete properdin system. Because serial complement titrations of tissue culture media indicated that little complement activity remained after 24 hours of incubation, cultures of HEp 2 cells were refed every 24 hours for 6 days. Duplicate cultures were grown in each of 5 media as follows: Complete P system (RP & R3 & P); P but no C′ (Heated RP & R3); C′ but no P (P heated); C′ but no P (P omitted); neither C′ nor P (RP & R3 heated, P omitted). Properdin when present titered 8 U/ml. Total C′ when present titered 40 U/ml initially and approximately 5 U/ml after 24 hours incubation. Heated preparations contained no detectable P or C′. All cultures grew well throughout the study without relation to presence or absence of C′ or P or both, as judged by daily microscopic observation of living cultures.

Exp. 3. Behavior of complement components and properdin in tissue cultures. To study rate of disappearance of C′ components and P, tissue culture medium containing a complete C′ system was incubated in bottles with and without growing HEp 2 cells and with and without P. Aliquots were removed after incubating for 2, 6, 24, and 48 hours. P showed no significant change. Total complement activity in bottles containing P fell rapidly during the first 6 hours of incubation and disappeared (<5 U/ml) by 24 hours. In bottles containing no P′ the fall-off of C′ activity was significantly slower, but C′ was undetectable by 48 hours (Fig. 1).

C′ 2 titers essentially duplicated the total C′ titer and thus C′2 appeared to be the limiting component at all times (Fig. 1). C′4 was not a limiting component initially but fell to levels nearly as low as C′2 by 6 hours. It dropped more rapidly in presence of P. C′1 was never a limiting factor (Fig. 1). In the presence of P, C′1 titer fell more rap-

idly than in absence of P, but it did not drop further after 24 hours. No drop of C'1 was demonstrated in bottles lacking P. Since C'1 levels were high and since titrations were based on dilutions in geometric progression, an absolute change in C' titers equal to that observed for C'2 would not have been detected (*e.g.* a decrease of C'1 from 240 U/ml to 200 U/ml would not be detectable by this technic, whereas an equal drop in C'2 would reduce it from normal levels to zero).

C'3 was never a limiting factor. It showed erratic fluctuations at 2 and 6 hours in bottles containing P or cells or both, but there were no significant differences at 24 or 48 hours.

As in all other studies, the cells grew well in all tubes throughout experiment.

Discussion. Under conditions of this study human properdin at physiologic concentrations had no cytotoxic effect. It follows that if serum properdin plays any part in the process of homo- and hetero-transplant rejection, a possibility suggested by several studies with human and animal systems(1,13,14,15), it must either require combined action with additional factors for its anticellular effect, or must act indirectly. The tissue culture media which were used contained all factors presently known to be necessary for reactivity of properdin in the zymosan assay (calcium and magnesium ions and the 4 components of complement). However, there is no reason to assume that these are the only components involved in other systems in which properdin may function. On the other hand, there is nothing in the experiments cited above which proves that properdin participates in homotransplant rejection. All observations could be equally well interpreted as indicating merely that properdin levels vary in parallel with some as yet undefined factors which *are* directly involved in rejection of transplanted cells.

The possibility that concentrations of properdin greater than 20 U/ml might have cytotoxic effects was not investigated, but seems irrelevant to the purpose of this investigation, since a concentration of 20 U/ml is well above the maximum normal level in human serum.

It is recognized that these cultures were not grown in complete absence of P, but merely at P concentrations below ½ U/ml. While it is conceivable that if cells were grown in complete absence of P, they might be adversely affected by addition of P, this possibility seems to have little application to the problem under study since in the previous clinical studies(1,6) homotransplant rejection was impaired when serum P levels were 2 U/ml or lower.

The fact that after a week of incubation in tissue culture 50% or more of original properdin content was still detectable, indicates that the cells were in contact with properdin throughout the entire study and that properdin is not rapidly metabolized by these cells.

Failure of growing cells to affect complement titers likewise indicates that complement is not rapidly metabolized by the cells. It also suggests that no reaction occurred between P and the cells, since it might be expected (by analogy with other properdin reactions) that if such a reaction occurred, complement would have participated in the reaction.

The rapid fall of C'2 and C'4 titers during incubation of the tissue culture media might be attributable to chemical lability without assuming any serologic reaction, but the fact that these titers fell even more rapidly when properdin was present, and that C'1 also fell in the presence of P, suggests that these 3 complement components may react with P even in the absence of a properdin-binding substrate. (The possibility of a P-binding substance in serum reagents is not excluded, but seems unlikely since it should have reacted *in vivo* or during the incubation steps in preparation of RP or R3.)

The erratic, but transient falls in C'3 titers in tissue culture media are unexplained. They suggest the possibility that C'3 may have reacted with properdin or with cells to form reversible complexes which made the C'3 temporarily inaccessible to the hemolytic detection system.

Conclusions. 1) Neither properdin (up to 20 U/ml) nor absence of detectable properdin ($<$½ U/ml) had any apparent influence upon growth rate or morphology of human cancer cells HEp #2 or J-111 in tissue culture, nor upon their ability to propagate on subsequent

homotransplantation. 2) Neither properdin nor complement was rapidly metabolized or inactivated by these cells in tissue culture. 3) Addition of properdin to tissue culture medium (in presence or absence of cells) caused a fall in C'1 titer and accelerated the fall of C'2 and C'4.

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Received June 1, 1959. P.S.E.B.M., 1959, v102.

Comparison in Rabbits of Hypoglycemia and B Cell Degranulation After Various Sulfonylureas.* (25145)

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It has been shown unequivocally that sulfonylureas produce degranulation of the pancreatic B cells of rats, rabbits and dogs when administered over prolonged periods(1-3). This degranulation has been interpreted(2) as indicative of suppression of insulinogenesis similar to that observed during chronic hypoglycemia induced by insulin administration. However, we demonstrated previously(4) that in rabbits insulin induced hypoglycemia did not produce degranulation, whereas hypoglycemia of equivalent depths and duration produced by tolbutamide caused varying degrees and frequently complete degranulation of the B cells. We undertook to determine whether the hypoglycemic sulfonylureas would produce pancreatic B cell degranulation even though their hypoglycemic action was prevented by simultaneous infusion of glucose. Furthermore, since the hypoglycemic effectiveness of these drugs apparently depends on their ability to increase insulin output from B cells and since this increased insulin output will be reflected in the extent of B cell degranulation, we attempted to correlate their blood sugar lowering efficacy with degree of B cell degranulation.

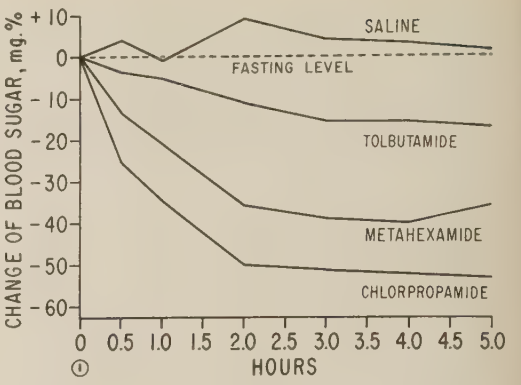
Material and methods. We used 72 New Zealand white rabbits of either sex, weighing 2.5 to 4 kg divided into 3 main groups. 1) *Single injections.* Eighteen normal rabbits, after overnight fast, each received intravenously 125 mg/kg of tolbutamide or chlorpropamide, or metahexamide. Furthermore, 6 animals received 1 cc/kg of saline intravenously and served as controls. Blood was withdrawn from marginal ear vein immediately before and at hourly intervals for 5 consecutive hours after administration of each test dose. 2) *Multiple injections.* Eighteen

* Supported by grant from Upjohn Co., Kalamazoo, Mich.

rabbits received intravenously twice daily 125 mg/kg of tolbutamide, chlorpropamide, or metahexamide for 28 consecutive days. Blood was withdrawn for glucose determination twice daily at 10 a.m. and 1:30 p.m. 3) *Infusion experiments.* Six rabbits were infused through marginal ear vein for 7 hours on 1, 2 or 3 successive days with approximately 300 cc of 5% glucose in saline. Additional 24 animals received a similar perfusate to which 625 mg/kg of either tolbutamide, chlorpropamide, or metahexamide had been added. An attempt was made to infuse a third group of animals with sulfonylureas in saline. This could not be accomplished since rabbits invariably expired during the first night. All animals were kept in individual metabolic cages and received Purina Rabbit Chow, carrots and water *ad lib.* Animals treated for prolonged periods received 200,000 units of Procaine Penicillin G and 0.25 g of dihydrostreptomycin sulfate (Combiotic Pfizer) twice weekly. Blood sugars were determined by Nelson-Somogyi micromethod(5). Rabbits were sacrificed by overdosage with barbiturates and the tail of the pancreas was immediately placed into Zenker-Formol (20%) solution for microscopic studies.

Results. A single injection of 125 mg/kg of tolbutamide caused a decline of blood sugar level which varied from –26 to –43 mg % (mean: –31 mg %). The nadir was obtained at 2 to 3 hours after which time blood sugar levels tended to rise. However, blood sugars did not regain the fasting level within 5 hours. Both chlorpropamide and metahexamide caused a more pronounced hypoglycemia. The maximum decline obtained with metahexamide varied from –33 to –57 mg % with a mean of –42 mg % while chlorpropamide decreased the blood sugar level which ranged from –46 to –66 mg % (mean: –57 mg %). In control experiments blood sugar concentration showed no significant fluctuations above and below the initial value (Fig. 1).

Multiple injections of 3 sulfonylureas produced a significant decline of blood sugars after each injection. However, the morning (non-fasting) levels were always within normal limits. No animals died in hypoglycemic shock.



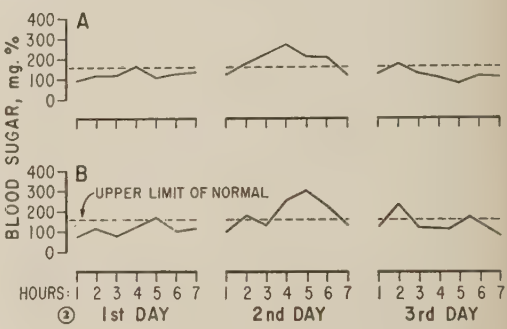


FIG. 1. Comparison of mean decline in blood sugar of rabbits after a single inj. of tolbutamide, metahexamide or chlorpropamide.

FIG. 2. Representative blood sugar curves of 2 rabbits: (A) infused through marginal ear vein for 7 hr on 3 successive days with 300 cc of 5% glucose in saline alone; (B) with a similar amount of glucose in saline to which 625 mg/kg (2 g) of metahexamide had been added.

Infusion of 300 cc of 5% glucose solution in saline for 7 hours produced the expected hyperglycemia with maximal blood sugar elevations obtained at 1 or 2 hours varying from 210 to 400 mg %. In animals in which the various sulfonylureas were administered simultaneously with glucose, degree of hyperglycemia was not significantly altered. Peak values ranged from 220 to 375 mg % (Fig. 2). In no instance was a significant decline of blood sugars below fasting levels observed.

A single injection of any sulfonylurea caused no alteration in pancreatic morphology. After chronic administration for 28 days there was usually similar extensive B cell degranulation with all 3 drugs.

Infusion of glucose alone for 3 days caused no detectable alteration in islet morphology. When sulfonylureas were added to the glucose perfusate varying degrees of degranulation

were obtained. Administration of chlorpropamide and metahexamide caused extensive degranulation after 2 days, which usually became almost complete after 3 days. On the other hand, perfusion of tolbutamide caused less pronounced degranulation after 2 days and only rarely complete loss of B cell granules even after administration of the drug for 3 successive days.

Discussion. It has been demonstrated that while daily infusion of glucose for 3 days causes no morphologic alteration of B cells, addition of various sulfonylureas to the perfusate produces distinct and usually complete B cell degranulation, despite the fact that glucose contained in the infusion is adequate to prevent reduction of blood sugar level. These findings are in keeping with our previous interpretations(4,6) that B cell degranulation is due to a primary action of the drug. They are also in accord with much clinical and experimental evidence which indicates that functioning B cells are a prerequisite for blood sugar lowering action of these drugs(1, 7-9). In further support of this interpretation is the present observation that the relative hypoglycemic effectiveness of equal dosages of these drugs is paralleled by the extent of B cell degranulation induced.

That glucose alone administered intravenously for 3 days caused no morphologic change in rabbit B cells is not in accord with the general impression based on studies in rats and guinea pigs(10,11) that glucose administration causes B cell degranulation. However, this finding is in keeping with previous demonstration that in rabbits made diabetic by administration of cortisone B cell degranulation is frequently not noted until 2 days after starting hormonal treatment(12). It is quite probable that administration of glucose immediately increases insulin output (13). This initial increase in insulin output is, however, not reflected morphologically in changes of pancreatic granulation. There is apparently a need for a sufficient extent and duration of hyperglycemia for B cell degranulation to become morphologically visible. This may be in accord with the previously expressed view(14) that the initial stage of B cell degranulation is a diminution in size of granules rather than a disappearance of granules and that morphologically visible degranulation only occurs when they have diminished very markedly in size.

Summary. 1) A single injection of same dose of 3 sulfonylureas, tolbutamide, chlorpropamide and metahexamide showed that tolbutamide caused a lesser hypoglycemia, whereas the 2 other drugs caused a more severe lowering of blood sugar level, most marked after administration of chlorpropamide. In no case was any B cell degranulation observed. 2) Infusion of glucose alone or simultaneously with various drugs for 7 hours each day on 3 successive days caused an identical hyperglycemic response in all animals. However, infusion of glucose alone produced no B cell degranulation; when sulfonylureas were added to the perfusate, degranulation of B cells was found. This was most pronounced after administration of chlorpropamide and metahexamide. That absence of hypoglycemia did not prevent B cell degranulation is further proof that degranulation of B cells after sulfonylureas is a primary action of these drugs on B cells to increase insulin output. Furthermore, relative B cell degranulating efficacy of the 3 compounds, in accord with their hypoglycemic effectiveness, supports the conclusion that they act directly on the B cell.

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Received June 17, 1959. P.S.E.B.M., 1959, v102.

Effect of Parathyroid Extract on *in vitro* Uptake of Ca^{45} by Voluntary Muscle.* (25146)

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For many years the majority of studies investigating mode of action of parathyroid hormone was undertaken to prove that it acted solely on bone or solely on kidney, with the result that a good deal of evidence has accumulated suggesting it acts on both(1). More recently, it has been suggested that parathyroid glands might also regulate transfer of phosphorus between extracellular and intracellular fluid(2,3). The thesis that parathyroid hormone can modify the passage of electrolytes across a membrane is implicit in earlier work of Clark(4), who, by addition of parathyroid extract, could almost completely inhibit *in vitro* uptake of calcium by the lens. The present experiment indicates that parathyroid extract (PTE) also inhibits *in vitro* uptake of calcium by muscle.

Materials and methods. Rats weighing approximately 150 g were used. They were sacrificed by blow on head, and abdominal wall musculature was excised and cut into equal segments. Incubating medium was prepared by adding to one liter of distilled water 9 g NaCl, 0.42 g KCl, 0.2 g $MgCl_2$, 1 g $NaHCO_3$, 0.05 g NaH_2PO_4, and 1 g glucose. $Ca^{45}Cl_2$ was added to give an activity level of 2000 cts/min/cc of incubating medium. The final concentration of calcium was 0.1 mg/liter. Twenty-five units (0.25 cc) of parathyroid extract (Eli Lilly Co.) was added to experimental flasks, and an equal volume of saline to control flasks. Each flask contained 6 cc of buffer at pH 7.4, and incubated in water bath at 37°C. Flasks were incubated unstoppered for 30 minutes and shaken manually every 5 minutes. Then the incubating medium was removed from both sets of flasks. Three 1 cc aliquots from each flask were pipetted into planchets, dried, and counted in Geiger-Müller counter. Values were expressed as amount of radioactivity remaining after incu-

TABLE I. Inhibition of Ca^{45} Uptake by Parathyroid Extract.

Flask No.	Ca^{45} activity remaining after incubation (cts/cc/min.)* Control	PTE added
1	1366	1644
2	1451	1611
3	1421	1621
Mean	1410	1623
1	1269	1574
2	1328	1544
3	1346	1498
Mean	1312	1536
1	1343	1693
2	1372	1676
3	1368	1685
4	1368	1641
Mean	1361	1672
1	1328	1446
2	1396	1568
3	1240	1598
4		1524
Mean	1319	1532
1	1423	1599
2	1487	1624
3	1470	1595
4	1270	1590
Mean	1411	1600
$t = 10.96$	$df = 4$	$p < .001$

* Initial conc. 2000 cts/cc/min.

*Authors are grateful to Dr. Glen Irwin of Eli Lilly Co. for generous supply of parathyroid extract, and express appreciation to Dr. Donald Korst, Radioisotope Service, Vet. Admin. Hospital, Ann Arbor, Mich., for laboratory facilities.

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bation with muscle. In early experiments each individual slice was weighed and counts/mg wet weight/cc/minute determined. However, it became apparent that weight variation was insignificant, and this was discontinued.

Results. Table I represents accumulated data from 5 experiments. Numerical values are the mean of 3 aliquots from each flask, and measured amount of radioactivity remaining after incubation. It is apparent that addition of PTE to the incubating medium consistently increased amount of radioactivity remaining and consequently must have inhibited uptake of Ca^{45} by muscle, or altered degree of absorption of Ca^{45} on the muscle cell membrane. Addition of PTE to the incubating medium in absence of muscle did not affect the final activity of Ca^{45}. The data were analyzed by Student-Fisher t test for significance of mean differences, and mean difference was significantly different from zero (P value $<.001$).

Summary and conclusions. Administration of parathyroid extract (PTE) elevates serum calcium(5). The present experiment indicates that PTE consistently inhibited *in vitro* uptake of calcium by rat voluntary muscle. This observation is compatible with the known hypercalcemic effect of parathyroid hormone and provides evidence for the hypothesis that parathyroid glands regulate movement of calcium between extracellular and intracellular fluid.

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Received June 17, 1959. P.S.E.B.M., 1959, v102.

Changes in Human Lung Collagen and Lipids with Age. (25147)

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Preliminary investigations indicate that collagen in human lung undergoes both quantitative and qualitative changes with increasing age(1). It is already known that a quantitative change occurs in animals; collagen content of guinea pig lung(2) and of rat lung(3, 4) increases with age. A qualitative change with advancing age has been demonstrated in collagen isolated from animal tissues other than lung. Collagen becomes increasingly stable with age when subjected to chemical and enzymatic degradation and to physical stress(5,6,7,8). While our investigations were in progress, it was reported that shrinking temperature (T_s) of collagen isolated from human skin increases with age(9). Differences in T_s among collagens from fish and bovine sources were reported by Gustavson (10). Imino acid analyses of these collagens led him to postulate that T_s is related to hydroxyproline content of the molecule, because collagens with the highest hydroxyproline content had the highest T_s(11). Hydroxyproline is thought to provide crosslinks between peptide chains by means of hydrogen bonds and thus to stabilize the structure of the protein and increase its resistance to stress. Our results concern both these quantitative and qualitative changes in collagen in human lung parenchyma and pleura. Concentration of collagen in lung parenchyma, the T_s of collagen of pleura, and the hydroxyproline content of collagen isolated from pleura have been studied and correlated with age. Observations of the lipid content of the lung are also included.

* Supported in part by grants from N.I.H., U.S.P.H.S. and the Polachek Fn.

Materials and methods. Samples of normal human lung parenchyma and pleura were ob-

tained at autopsy in Bellevue Hospital. The term pleura includes the serous membrane, subpleural connective tissue and probably small amounts of attached underlying parenchyma. Parenchyma was selected from peripheral areas of the left upper lobe. These specimens were analyzed as follows: 1. Lung parenchyma from 8 women and 18 men, aged at time of death from 28 to 84 years, was analyzed for total lipids. Lipids were determined by weighing ether soluble extract obtained from an aliquot of whole dried tissue by the Soxhlet method. 2. Lung parenchyma from 8 women and 19 men varying in age from 27 to 77 years was analyzed for hydroxyproline by the colorimetric method of Stegemann(12). Quadruplicate samples of fresh tissue weighing 200 to 300 mg were placed in vials with 6 N HCl and sealed. The vials were heated at 110°C for 48 hours. Hydroxyproline was determined in aliquots of neutralized, diluted hydrolysate thus obtained. Results are reported either as per cent hydroxyproline or as per cent collagen of dry weight of tissue. Percentage dry weight was determined in another specimen of each lung by drying to constant weight in oven at 110°C. 3. The T_s of strips of pleura from 7 women and 20 men 20 to 81 years old was measured in distilled water. Strips of pleura were prepared as described by Gustavson for fish and bovine skin (10). For measurement, the strip was attached under slight tension with thread to a lever and submerged in beaker of water heated at the rate of about 1°C/min. The T_s was recorded as that temperature at which the lever began to move. 4. Hydroxyproline content of collagen, isolated as gelatin from pleura of 14 males and 10 females, from 10 weeks to 77 years old, was determined. Gelatin was isolated by a method essentially similar to that of Neuman and Logan(13). Hydrolysis of gelatin and determination of hydroxyproline was done as described above. The results are reported as grams of hydroxyproline/100 g of gelatin.

Results. Lipid concentration of dried lung parenchyma, plotted as a function of age, is presented in Fig. 1. The hydroxyproline concentration of lung parenchyma is similarly plotted against age in Fig. 2, and expressed as per cent of hydroxyproline or collagen of the dry weight, assuming former to be 13.5% of the latter(13). The average values of lipid, collagen and elastin/decade of adult life used to calculate these graphs are shown in Table I. Correlation between T_s of pleural strips and age is illustrated in Fig. 3 ($t = 7.24$; $p < 0.01$). Results of analyses of collagen isolated as gelatin from these pleural strips are presented in Fig. 4.

TABLE I. Average Concentrations in Human Lung of Collagen, Lipid and Elastin per Decade of Adult Life.

	Collagen*		Lipid	Elastin†
Age, yr	% dry wt	% fat-free dry wt	% dry wt	% dry wt
25	8.7	9.7	10.1	6.2
35	9.7	10.7	9.2	7.0
45	10.8	11.8	8.3	7.8
55	11.9	12.9	7.4	8.6
65	12.9	13.8	6.5	9.5
75	14.0	14.8	5.5	10.3
85	15.0	15.7	4.3	11.1

* Values corrected for elastin.
† Taken from Fig. 1 in previous report(14).

Discussion. Hydroxyproline concentration of human lung increases with age both on the basis of total dry weight and of fat-free dry weight ($t = 4.33$; $p < 0.01$). A further correction for the inhaled foreign material found in increasing amounts in aging lungs would increase steepness of slope of the regression line. The question arises, whether or not this increase in hydroxyproline content represents increase in collagen concentration. If the amount of hydroxyproline due to lung elastin is subtracted (Fig. 2, dotted line), using values for elastin obtained in an earlier series of experiments(14), presumably the remainder is present only in collagen. This assumption is based on the hypotheses that no other protein contains hydroxyproline, and that hydroxyproline is not free in the tissues but arises within the protein molecule from proline(15, 16). If the hydroxyproline content of collagen in lung parenchyma increases with age, it is conceivable that an increasing hydroxyproline content of lung parenchyma (Fig. 2) could be compatible with a constant collagen content at all ages. This could not occur, however, unless the hydroxyproline content of collagen increased with age about 6 times as

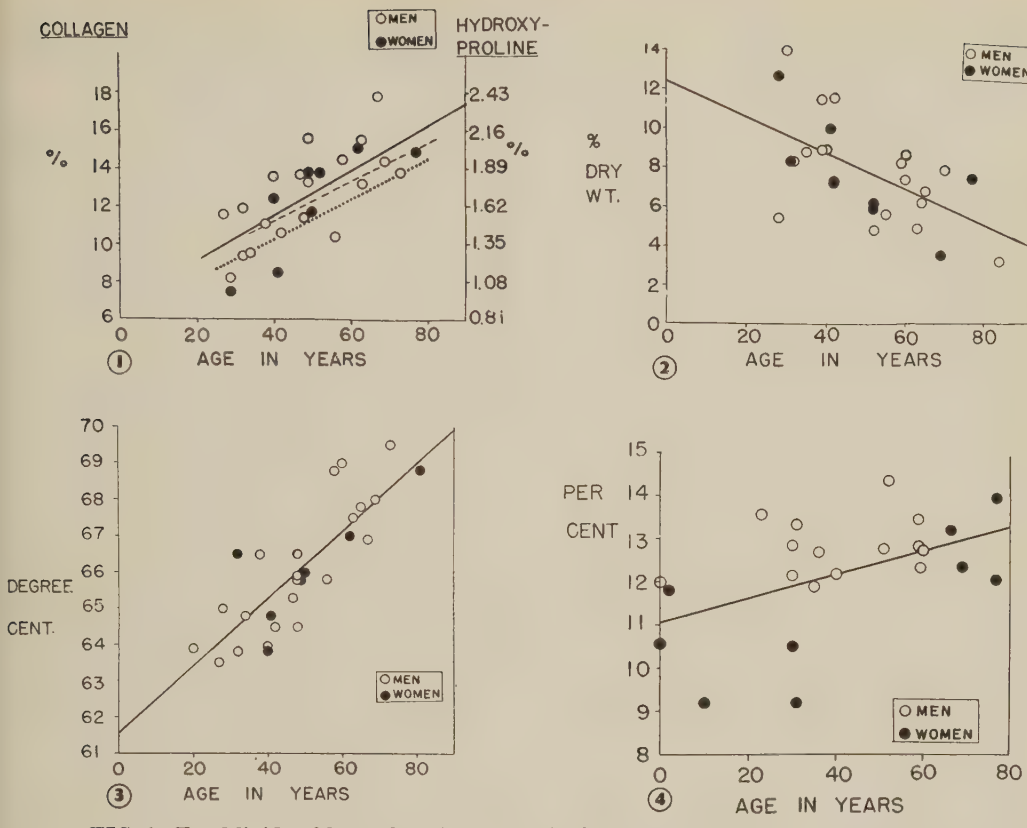

FIG. 1. Total lipids of lung plotted *vs* age: % of dry wt of tissue.

FIG. 2. Collagen or hydroxyproline concentration of lung plotted *vs* age. Solid line: total hydroxyproline as % of dry wt of tissue. Broken line: collagen, corrected for elastin, as % of fat-free dry wt. Dotted line: collagen, corrected for elastin, as % of dry wt.

FIG. 3. Shrinking temperature of pleura plotted *vs* age.

FIG. 4. Hydroxyproline concentration of pleural collagen plotted *vs* age: % of dry wt of collagen isolated as gelatin.

much as in pleura. Other workers whose results were based on alkali extraction method rather than amount of hydroxyproline found that the collagen content of rat and guinea pig lungs increased similarly with age(2,3,4) and the magnitude of their values was of the same order as that reported here. The data of Fig. 4 obtained from collagen from pleural strips were not applied to the data in Fig. 2 which concerns lung parenchyma. Pleura and parenchyma have different structures and might contain collagens of different hydroxyproline concentrations. A study of collagen isolated from lung parenchyma should precede the assumption that it is identical to that of pleura.

Lipid in the lung is not present in the form of adipose tissue. Thus, the practice of defatting tissue as a preface to analysis for other substances, which may be justified in the case of tissues containing variable amounts of adipose tissue, is not valid here. Results obtained with defatted tissue do not reveal the proportion of lung which is collagen. Other workers reported that collagen and elastin content of defatted, dried lung remains constant through life(17). Our data indicate, on the contrary, that even in fat-free lung, the increase in hydroxyproline concentration with age still obtains (Fig. 2, broken line). In addition, our results correlate lipid content of lung with age ($t = 3.42$; $p<0.01$). The amount of lipid in the lung is apparently not a function of body size or nutritional state since the specimens were obtained from a random selection of individuals with regard to these physical

and physiological characteristics.

Analogous to work with skins, the assumption has been made here that T_s of a pleural strip is due to contraction of its collagen fibers. Contraction of collagen fibers on heating is sharp and characteristic under identical circumstances. Thus values obtained here for collagen under a slight tension are higher than those reported for isolated fibers from human skin which were allowed to float free(9). This is consistent with Gustavson's comparison of freely suspended fish skins with those fastened to a shrinkage measuring device like ours(10). Our data agree with those of Brown and Consden on human skin(9) insofar as age change is concerned and are in contrast to those of Hall and Reed(18) who found no age difference in T_s of isolated human collagen in water. However, the measurement of T_s of collagen in tissue may be more valid because the isolation procedure may alter the collagen molecule.

Correlation between increasing hydroxyproline concentration and increasing age was significant ($t = 2.44$; $p<0.025$). If the 2 lowest values of 9.2% each are considered questionable and prejudicial, they may be excluded; the statistical significance of the data is not impaired thereby. The average value of hydroxyproline concentration in collagen isolated from 21 adult specimens was 12.4%. The range was from 9.2 to 14.4%. The data in Fig. 2 and Fig. 3 suggest that collagen of women has a lower shrinking temperature and lower hydroxyproline concentration than that of men, but a larger number of observations is needed to confirm this.

Although some correlation between T_s of collagen and its hydroxyproline content has been demonstrated, there are collagens from widely different sources which do not conform to the scheme(19,20). That T_s of invertebrate collagen is higher than that of vertebrate collagen of similar hydroxyproline concentration suggests that some other feature of collagen structure contributes to its hydrothermal stability. It may be invalid to compare collagens of invertebrates with those of vertebrates. A study of aging invertebrate collagen may show a relation between the 2 properties. Among collagens of similar composition, variations in hydroxyproline concentration appear to produce corresponding changes in T_s.

Summary. 1) Hydroxyproline content of human lung increases with age. 2) The collagen content of human lung increases with age. 3) The lipid content of human lung decreases with age. 4) The shrinking temperature of human pleura increases with age. 5) The hydroxyproline content of collagen isolated from human pleura increases with age.

The authors greatly appreciate the support and interest by Dr. Robert F. Loeb, and the valuable advice of Drs. Walter Troll and W. A. Briscoe.

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Received June 19, 1959. ·P.S.E.B.M., 1959, v101.

Adsorption of Insulin to Glass.*† (25148)

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Several investigators have shown that I^{131} insulin is adsorbed to glassware(1,2). The reversibility of this phenomenon has been demonstrated(3). If non-iodinated insulin behaves in a similar manner, those working with small amounts of insulin, such as are used in micro-insulin assays designed to measure circulating insulin, must take precautions to avoid errors owing to losses and contamination because of adsorption and desorption. This report describes experiments demonstrating the adsorption of non-iodinated insulin to glass and its desorption from glass.

Materials. Crystalline zinc insulin was used. Acidified saline was made by adding 1 ml concentrated HCl to 2 l 0.9% NaCl. All glassware used was washed and then rinsed with 5 N or stronger NaOH to destroy any insulin adsorbed to it. It was rinsed in distilled water to remove the alkali. Powdered Pyrex glass which had been through a 200 mesh screen was placed on a medium porosity sintered glass funnel. The funnel containing the powdered glass and a stirring rod was allowed to stand overnight in a solution of 5 N NaOH or concentrated HNO_3. They were then washed with distilled water and dried in oven at 120°C. *Determinations of hypoglycemic activity* were carried out in 2 groups of hypophysectomized mice. The North Carolina mice have been inbred since 1941 at the State Laboratory of Hygiene, Raleigh, N.C. The other group used were mice of the A stock. Hypophysectomy and postoperative treatment were performed according to the method of Lostroh and Jordan(4). The morning of the experiment the hypophysectomized mice were placed in individual cages without food or water. About ½ hr later the first blood sample was taken and appropriate gelatin solution was injected intraperitoneally in volume of 0.25 ml. The mouse was returned to its individual cage. At 60 min. after injection the second blood sample was taken. Blood was collected from the cut tip of tail in tared capillary tubes. The weighed sample of blood (5 to 10 mg) was rinsed out of the tube into 0.5 ml of water and proteins were precipitated according to the method of Somogyi(5). After centrifugation a measured volume of the supernatant was used for blood sugar determination, which was done either by the method of Park & Johnson (6) or a modification of the glucose oxidase method(7). In the hypoglycemic convulsion experiments mice which had been starved for 20 hr were placed in groups so that each group had a similar distribution of mice of varying weights. Solutions were injected intraperitoneally and the mice kept at 37°C.

Results. Table I summarizes 4 experiments in which 1 ml acidified saline containing crystalline insulin was diluted to 1000 ml with acidified saline or acidified saline containing 5% U.S.P. gelatin in 1000 ml glass stoppered graduated cylinders. The solutions were mixed by inverting the cylinders several times. The cylinders were allowed to stand as noted in Table I. The contents were poured out and the vessel allowed to drain for short time, after which it was weighed. The increase over the dry weight of vessel was used as a measure of solution which did not drain. The cylinders used in experiments B, C, and D were then filled with 1000 ml of distilled water and tipped several times. The water wash was discarded and the vessel again weighed. The amount of insulin in the solution which did not drain was calculated. Fifty ml of 5% U.S.P. gelatin in acidified saline was then placed in each of the 4 cylinders, which were stoppered and placed horizontally

* This investigation supported in part by research grant from Natl. Inst. of Arthritis and Metabolic Diseases of N.I.H., P.H.S.

† The author thanks Dr. O. K. Behrens, Eli Lilly Research Labs for crystalline insulin; Carolyn May and Helen Johnston for technical assistance; and Drs. Edmund Gehan and Earl Diamond, Dept. of Biostatistics, Univ. of N. Carolina, for statistical evaluation of data.

TABLE I. Hypoglycemic Activity Recovered from Walls of Graduated Cylinders.

Exp.	A	B	C	D
Units crystalline zinc insulin placed in cylinder	2	25	25	25
Diluent	acidified saline	acidified saline	acidified saline	5% gelatin
Time and temp. of storage	30 min. room temp.	35 min. room temp.	6 days 5°C	6 days 5°C
No. times emptied*	1	2	2	2
No. hypophysectomized mice used in exp.	15	18	18	18
Mice	N.C.	A	A	A
% fall in blood sugar				
Gelatin from cylinder	18.2	14.8	11.4	−1.6
Control gelatin	−3.3	−2.1	0.4	0.4
P†	.02	.04	.001	.45
Estimate of max amt insulin which could have been inj. into mouse owing to non-drainage (microunits)‡	20	.5	.1	1.8

* In exp. B, C, and D, 1000 ml distilled water were added to cylinder after first emptying. The cylinder was tipped 40 times and emptied a second time.

† Exp. C and D were done as a 3 × 3 Latin Square. These probabilities are the result of carrying out 2 independent comparisons available within two degrees of freedom for differences between 3 treatments.

‡ These estimates are based upon weight of dry cylinder and weight of cylinder after drainage of various solutions. Our hypophysectomized mice do not give significant hypoglycemic responses until doses of the order of 250 microunits/mouse are given.

on a metal lathe and turned slowly for one-half hour. The gelatin solutions from the cylinders were compared with samples of the same gelatin solutions which had not been in the cylinders with respect to their hypoglycemic effects in hypophysectomized mice. In every case where the initial dilution of the insulin was made with acidified saline not containing gelatin (experiments A, B, and C) significant hypoglycemic activity appeared in the final gelatin wash. When the amount of insulin which could have appeared in this wash owing to lack of drainage of solution from the cylinders during the experiment was calculated, it was always considerably less than the amount which causes hypoglycemia in hypophysectomized mice. When the initial dilution of insulin was made in the presence of 5% gelatin (D), significant amounts of hypoglycemic activity did not appear in the final gelatin wash. When the per cent fall in blood sugar elicited by the gelatin from the cylinder in Exp. C of Table I (11.4) was compared with that of Exp. D (−1.6), the probability of the difference being due to chance variation was less than 0.001. This indicates 5% gelatin inhibits adsorption to glass of the hypoglycemic material.

Table II summarizes data from 2 experiments in which insulin in acidified saline was stirred with 5 g of powdered glass in a sintered glass funnel. The solutions were allowed to remain in contact with the glass from 1 to 2 hours with occasional stirring, then sucked through the filter into graduated cylinders. An acidified saline wash was placed on the glass and after thorough stirring sucked into a graduated cylinder. Ten ml of 5% U.S.P. gelatin in acidified saline was then stirred with the glass and allowed to remain in contact with it for 24-48 hours in a refrigerator. After the glass-gelatin mixture was liquefied by warming, it was centrifuged since it was too viscous for filtration. The supernatant gelatin was injected into mice as indicated in Table II. On the basis of volumes of solution recovered a calculation was made of maximal amount of insulin which could have remained on the glass at each step of experiment if no adsorption had occurred. In both experiments the gelatin solution which had been in contact with powdered glass caused hypoglycemic convulsions while insulin added to gelatin in amounts greater than would have been expected owing to lack of drainage of solutions from the powdered glass

TABLE II. Hypoglycemic Convulsions Produced with Insulin Eluted from Powdered Glass.

10 ml insulin sol. in contact with glass			10 ml acidified saline wash		10 ml 5% U.S.P. gelatin			Control insulin in 5% gelatin		
						Gelatin from glass				
Conc., μg/ml	ml recovered	μg left on glass	ml recovered	μg left on glass	Max not draining, μg/ml	ml	Mice in convulsions	μg/ml	ml	Mice in convulsions
30	9.4	18	10.2	1	0.1	1.0	3/3	4	1.0	0/3
						.3	1/3			
35	9.0	35	10.2	2.5	0.25	.25	15/17	4.5	.25	10/10
						.10	2/10	4.5	.10	3/10
								.5	.25	0/17

did not cause convulsions. Evidence that the convulsions were hypoglycemic was obtained by administering 10% glucose intraperitoneally to mice in convulsions and terminating the convulsions. Three mice bled during convulsions had blood sugar values below 45 mg %. To obtain some idea of quantity of insulin eluted from the glass by the gelatin, a solution of 4.5 μg/ml insulin diluted with 5% U.S.P. gelatin in acidified saline was administered in the same dose ratio as the gelatin from glass in the second experiment of Table II. The results are similar to those obtained when gelatin from the glass was used. This suggests that the gelatin from the glass contained several μg/ml. The difference in insulin sensitivity of the mice used in the 2 experiments of Table II is probably owing to size and strain difference.

Conclusions. Our results indicate that material having hypoglycemic activity is adsorbed to glass from a solution of insulin and may be eluted from it with a solution of 5% U.S.P. gelatin. Since adsorption of proteins to glass is a well known phenomenon and since the hypoglycemic activity of insulin has never been dissociated from the intact molecule, it is safe to assume that insulin is adsorbed to glass and may be eluted from it. Under proper conditions a 5% solution of gelatin will inhibit adsorption of insulin to glass.

Summary. By elution with gelatin solutions a material having hypoglycemic activity in mice can be recovered from laboratory glassware which has previously been exposed to insulin. Gelatin solutions can prevent adsorption of insulin to glass.

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Received June 19, 1959. P.S.E.B.M., 1959, v102.

Choriocarcinoma of Women Maintained in Serial Passage in Hamster and Rat. (25149)

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The heterologous transplantation of malignant tumors of man was initially reported by Greene(1). Toolan(2) and Sommers *et al.* (3) described methods for increasing the frequency of success of such heterologous tumor grafts through prior suppression of the immune response of the animal host by x-irradiation and by cortisone administration. Pierce *et al.* described the biological behavior of human testicular tumors carried in the hamster

cheek-pouch(4,5). We employed similar procedures in the adaptation of 3 choriocarcinomata of women to serial transplantation in the hamster. One of these tumors has now also been maintained in serial transfer in the rat. This report will describe the biological and hormonal features of these tumor strains. We named our tumors the Erwin-Turner Choriocarcinoma,* Strains WO, BO, and MA.

Materials and methods. Tumor tissues used were derived from 3 women with a histopathologically established diagnosis of choriocarcinoma according to morphological criteria of Novak and Seah(6). The original tissues for strains WO and MA were obtained at autopsy from cerebral metastases. Strain BO was derived from a nodular metastasis which had been surgically resected from patient's right breast. All 3 patients had shown an initial therapeutic response to methotrexate(7,8). However, patients who provided tissue for strains WO and MA had previously become methotrexate-resistant. The patient who provided the tissue for strain BO continued to show a limited degree of response to methotrexate. Female golden hamsters of NIH strain varying from 1-3 months in age were employed. Intact and hypophysectomized female Sprague-Dawley rats weighing 45-55 g were also used. All animals were maintained at room temperature between 76° and 80°F. Purina checkers were fed as basal diet. Hypophysectomized rats received ground-meat supplement daily and hamsters received daily ration of kale, carrots, and apples. Starting with original human tissue, transfers have been made by inoculation into the hamster cheek-pouch by means of freshly excised piece of tissue about .05 cu cm in volume. Rat transfers have all been made subcutaneously in the right flank area. Aseptic precautions have been applied throughout. Nembutal anesthesia was employed. Recipient hamsters were given 3 mg of cortisone acetate in aqueous suspension at time of inoculation and every third day subsequently during ensuing 2 weeks. Recipient rats received the same dosage schedule of cortisone for a 3 week period and were also exposed to general body irradiation just prior to inoculation, as recommended by Toolan(2). Repeated appraisal of qualitative and quantitative development of tumor inocula were made by direct inspection and by free-hand sketch of size and shape of enlarging tumor mass. Histological studies were performed on selected specimens by fixation in Bouin's solution, paraffin sectioning and staining with hematoxylin and eosin. At autopsy, careful evaluation of quantitative and qualitative hormonal effects of tumor

TABLE I. Transfer Generations of WO Strain of Erwin-Turner Choriocarcinoma in Hamster and Rat.

Transfer generation	Date of transfer	No. of animals used	No. of takes after 7 days	Recipients
1	10/24/58	20	15	Cortisonized hamsters
2	11/10	35	22	
3	26	35	19	
4	12/18	50	24	
5	23	20	17	
6	1/ 8/59	80	52	
7	1/13/59	33	18	Hamsters (no prior treatment)
8	22	25	20	
9	30	40	32	
10	2/ 9	20	13	
11	16	25	20	
12	24	25	14	
13	3/ 4	25	16	
14	11	20	17	
15	19	30	25	
16	26	72	49	
17	4/ 2	21	18	
18	9	24	16	
19	16	46	39	
20	23	59	42	
21	30	24	17	
22	5/ 7	42	36	
1	10/24/58	10	3	Rats (cortisonized and irrad.) hypophysectomized (H) or intact
2	11/10	20	4	
3	21	20	6	
4	12/10	30	10	
5	20	20 (H)	10	
6	1/ 6/59	20 "	12	
7	21	10 "	6	
8	28	12 "	7	
9	2/ 6	22 "	10	
10	20	11 "	4	
11	3/ 3	20 "	12	
12	13	20 "	11	
13	25	22 "	9	
14	4/ 3	17 "	7	
15	14	22 "	10	
16	24	22 "	10	

* These tumors are named in grateful acknowledgement of efforts of Mr. Howard L. Erwin and Mr. Charles K. Turner without whose diligence and technical skill this valuable material would not have become available.

transplant upon various endocrine organs were recorded.

Results. There are listed in Table I the actual transfer generations of 1 of the tumor strains (WO). Time intervals may be calculated from dates of respective transfers. The almost immediate adaptation of this strain to the heterologous host may be noted. Transfers were usually made when the tumor had reached an estimated volume of 1-1.5 cc.

Growth of each tumor strain has assumed a characteristic course. The WO strain has proven to be the most readily adaptable strain in both hamster and rat. It quickly exhibited a high percentage of takes and a very rapid course. Transfers are now readily made at weekly intervals and the tumor runs its full course to spontaneous necrosis and liquefaction in 15-20 days. Moreover, it has now been carried in serial transfer for 12 generations in the hamster without use of cortisone. This makes the strain especially useful in studies of endocrinological relationships. In the rat, however, the WO strain still failed to grow unless the host had been irradiated and had also been treated with cortisone. In addition, tumor growth in the rat was considerably slower and spontaneous necrosis occurred between 10-15 days following inoculation.

In the WO strain the initial phase of host response is noted in hamster cheek-pouch within 2-3 days. This consists of formation of a turbid exudate about the tissue inoculum. In the following 3 days this is rapidly replaced by highly vascularized, bluish-purple tissue which then extends and rapidly fills the cheek-pouch. Maximum size of 1-1.5 cc is usually reached by about 10 days and during ensuing 5-10 days the tumor loses its bluish color and becomes successively pink, grey, and finally greenish yellow. At this point, the necrotizing mass may slough and drain and by 30 days following inoculation, only a small residual scar may be found in the cheek-pouch. Less frequently the liquefied tumor mass is indefinitely retained. In this strain 60-80% takes are regularly noted at one week following inoculation and an additional 10-15% of inoculated animals will develop a growing tumor during second week.

The remaining 2 strains, BO and MA, continue to require cortisone for survival. They also have a smaller percentage of takes and their growth progress is about ⅓ as rapid as that observed in the WO strain. Nevertheless, their qualitative behavior is quite comparable in all respects to that already described for the WO strain. It seems that heterologous adaptation of these 2 strains is not yet as complete as is that of the WO strain. The BO strain has been transferred 28 times in 22 months and the MA strain has been transferred 12 times in 7 months.

Histological study of all 3 strains reveals a close identity of the morphology of the heterologous transplant with that of the original patient material. The rich vascularization of tissue is its most noteworthy feature. The marked tendency to hemorrhagic necrosis of extensive areas of the tumor is consistently observed. Cytotrophoblast and syncitial trophoblast are formed into broad sheets and abundant mitoses are observed throughout. Marked cellular pleomorphism is characterized by very frequently occurring bizarre, multinucleated cellular and acellular masses. On the whole, the picture represents one of rapid, disorganized growth with extensive hemorrhage and necrosis.

Nevertheless, in no instance have we observed microscopic evidence of actual cellular infiltration into the wall of the hamster cheek-pouch. Moreover, no instance of distant metastasis has been observed. It is especially noteworthy that this highly proliferative and densely vascularized tumor tissue readily peels away from the surrounding host tissues with practically no hemorrhage at points of separation.

The peripheral endocrinological effects of the 3 tumor strains in the hamster are quite comparable (Table II). The ovaries of recipient animals show extreme gonadotropic stimulation characterized by 3-5 fold enlargement consisting of marked follicular and luteal stimulation. Their uteri, Fallopian tubes, and vaginae are extremely hyperemic and grossly enlarged. That this effect is secondary to the ovarian stimulation is shown by total absence of such effect in the previously ovariectomized recipient. Hence it may be inferred that these tumor strains are not directly productive of

TABLE II. Endocrine Effects of Human Choriocarcinoma in Female Hamsters and Rats.*

Species	Status	Tumor	No. of animals	Body wt	Ovaries	Uterus	Adrenals	Thyroid
				(mg)				
Hamster	Intact	+	25	73 ± 8	143 ± 30	641 ± 80	12 ± 2	
		—	10	82 ± 5	27 ± 3	276 ± 76	10 ± 1	
	Ovariecto-mized	+	30	102 ± 6		32 ± 6	10 ± 2	
		—	10	100 ± 7		30 ± 4	10 ± 1	
Rat	Intact	+	15	65 ± 9	230 ± 40	312 ± 80	23 ± 6	5 ± 2
		—	6	50 ± 4	10 ± 2	18 ± 2	9 ± 3	3 ± 2
	Hypophysec-tomized	+	18	49 ± 5	44 ± 6	83 ± 12	10 ± 2	3 ± 1
		—	10	50 ± 4	6 ± 1	17 ± 3	9 ± 2	3 ± 1

* All values are for Erwin-Turner choriocarcinoma, Strain WO. All rats were previously cortisonized and irradiated. All hamsters previously untreated; data are from one representative series.

estrogenic steroids in the heterologous host (Table II). The tumor-bearing hamster also exhibits no gross or microscopic evidence of thyrotropic, lactogenic or adrenotropic effect.

Similarly, the WO tumor strain exhibits only gonadotropic action in either the hypophysectomized or intact rat. Lactogenic, adrenotropic, or somatotropic potentialities cannot be readily evaluated in the rat because of complicating peripheral action of high doses of cortisone given these tumor recipients. Thyrotropic effect is entirely lacking. The marked quantitative and qualitative difference in gonadotropic effect in the hypophysectomized rat versus the intact rat indicates that the gonadotropic action of this tumor is entirely comparable with that considered to be characteristic for human chorionic gonadotropic hormone(9) (Table II).

Gonadotropic potency can be demonstrated in homogenates of heterologously maintained tumor tissue as well as in the peripheral blood of the tumor-bearing hamster by the use of infantile mice as test animals. As little as 2 mg of fresh tumor tissue or 1 cc of peripheral blood will induce maximum uterine enlargement and ovarian stimulation in an infantile mouse. Further studies will be required for a more detailed quantitative estimate of the actual hormonal output of these several tumor strains.

The growth pattern of each respective tumor strain has proven to be highly reproducible from generation to generation. Our method of recording of these growth processes by the use of free-hand, life-size, sketches of the tumors at various time intervals is admittedly crude. Nevertheless, these growth-records are sufficiently quantitative to permit gross evaluation of the effect of various inhibitory agents upon the course of tumor growth.

Summary. 1) Three choriocarcinomata from women have been successfully adapted to serial transplantation in cheek-pouch of the cortisonized hamster and one of these can be carried in previously untreated hamster. This latter strain has also been adapted to subcutaneous growth in the cortisonized, irradiated, hypophysectomized or intact female rat. 2) These heterologously maintained tumors produce in the host gonadotropic effects characteristic of human chorionic gonadotropic hormone. Biologically detectable amounts of hormone are readily demonstrable in homogenates of the growing tumor tissue and in peripheral blood of tumor-bearing hamster. The tumors exhibit no estrogenic, adrenotropic, or thyrotropic effect in the hamster. 3) Each tumor strain presents a quantitatively reproducible growth pattern which renders it adaptable to studies of the effect of chemotherapeutic and other inhibitory agents.

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Sensitivity of Populations of Clonal Lines of HeLa Cells to Polioviruses.* (25150)

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Fluctuations in growth potential and susceptibility to invasion by a given virus in HeLa cell "farm" populations have been a common experience. In addition to these changes, we have also experienced emergence of cells which had a rapid growth potential and which failed to take up neutral red adequately for the detection of plaques. Our own experience with these periodic changes suggests that they occur without any known alterations in environment which might select certain types; the degree of change at times necessitated interruption of our studies. In an attempt to obtain HeLa cell lines which exhibit greater stability of growth potential and virus susceptibility, populations were propagated from single clones isolated by the method of Puck (1). Six presumably clonal lines and a number of subclonal lines were isolated from a "farm" population of HeLa cells and examined for their sensitivity to types I and II polioviruses. The cell lines were found to differ in their sensitivity; two of the most sensitive have been maintained for two years and the above fluctuations have not recurred to an appreciable degree. Variation in susceptibility to poliovirus among clonal strains of HeLa cells has been reported and the variation appears to be a stable character(2).

Materials and methods. "Farm" population. The "farm" HeLa cell population, originally obtained from Dr. Scherer, has been carried with weekly passage in a variety of media. The medium used for the present study is that modified by Puck, *et al.*(3). It was sterilized by Seitz filtration. *Isolation of clonal lines.* The technic of isolation of single clones was that described by Puck, *et al.* 24-hour old subcultures of the cells were trypsinized for 10 or more minutes (until populations were comprised mostly of single cells) and, after appropriate dilution in the growth medium, were plated in 60 x 15 mm Petri dishes. The plate preparations, placed in containers that could be made air-tight, were gassed for a few minutes with 5 per cent CO_2 in air and then incubated at 37°C for 9 to 11 days. In estimating the percent of single cells in trypsinized suspensions, clumps of no more than 2 cells were considered to be incompletely divided cells and were grouped as single cells. On this basis, 90 per cent or more of the populations plated were single cells. Some of the trypsinized parental clonal populations used for the isolation of subclonal lines were virtually 100 per cent true single cells. The plating efficiency of all but one of the parental clonal lines at the time of subclonal isolation was between 30 and 65 per cent. Plating efficiency *per se* was not explored for the different clonal populations. Colonies of cells were selected for isolation on the basis of gross microscopic morphology and adequate separation from adjacent colonies. Selected clones were ringed by cylinders as described by Puck *et al.*, trypsinized, and then transferred to 16 x 150 mm test tubes containing 1 ml of growth medium. After adequate growth on the glass, the cells were transferred to 2-oz bottles in 3 ml of medium and subsequently to 5-oz bottles in 10 ml of medium. In general, single clone populations were first tested for

* This work was supported by grants from Natl. Inst. of Health.

their sensitivity to the polioviruses within 1 month of their primary isolation. *Test for sensitivity to polioviruses.* Clonal lines were tested for their sensitivity to types 1 and 2 polioviruses by the plaque assay technic. Approximately 1,000,000 cells of trypsinized week-old cultures of each cell line were used to seed 60 x 15 mm Petri dishes containing 4 ml of growth medium. The suspensions were then gassed for a few minutes with 5 per cent CO_2 in air and incubated at 37°C for 3 days in an airtight container. After incubation, the cell monolayers were washed twice with a Tris-buffered balanced salt solution (pH approximately 7.2) and inoculated with 0.1 ml of a dilution of the virus. After one half hour at 37°C, 4 ml of an agar overlay(4) was added and the plates were incubated for 4 days. 4 ml of a second agar overlay(4) containing neutral red was then added, and the number of plaques recorded after 4 to 5-hours incubation at 37°C. *Polioviruses used to test sensitivity.* Type I (Mahoney) and type 2 (MEF-1) polioviruses, originally obtained from the Connaught Labs (Toronto) were grown on "farm" populations of HeLa cells and diluted in Tris-buffered balanced salt solution before plating on monolayers. For most experiments, aliquots of a single preparation of each type of virus were used.

Results. Selection and sensitivity to polioviruses of parental clonal lines. Six single colonies of cells were selected from the "farm" population on the basis of morphology. The predominant type consisted of cells that were spread out so that a compact colony was not formed; 4 of this general type (clones 1, 2, 3 and 6) were isolated. Clone 4 was distinguished by its unusually transparent appearance. It gave rise to populations which in early passages were more transparent than those of the other cell lines, and which tended to grow in groups arrayed in a parallel fashion. With further passages, these characteristics were not constant; the cells assumed the gross morphology of the predominant clonal types. Clone 5 was clearly distinguishable from the others since it produced a much denser colony and was composed of cells that appeared small and were closely adherent; on subculture the cells tended to grow in circumscribed ovals or circles. Patches of this type of growth were noted in the "farm" population. Clone 6 on subculture appeared to contain a high proportion of spindle-shaped cells in young cultures.

TABLE I. Sensitivity of Populations of Clonal Lines of HeLa Cells to Types 1 and 2 Polioviruses.

Clonal line	Avg No. of plaques formed/monolayer			
	Type 1 virus		Type 2 virus	
	Exp. 1	Exp. 2*	Exp. 1	Exp. 2*
1	26	21	116	77
2	11	13	16	41
3	12	13	63	68
4	2	12	1	1
5	92	76	app. 280	app. 300 †
6		39		112

Each experiment (Exp.) represents simultaneous testing of all cell lines.

Aliquots of same virus preparation and same dilution used for each exp.

* Performed 2 wk after Exp. 1.

† app. = approximate.

These clonal lines were tested for their sensitivity to types 1 and 2 polioviruses approximately 1 month after their primary isolation. Monolayers of each clonal line were simultaneously seeded in triplicate with 0.1 ml of an appropriate dilution of each type of virus and treated as described under "Materials and methods." Two weeks later the experiment was repeated. Table I lists the average number of plaques formed per monolayer with each cell line and virus. The distribution of the number of plaques formed in each triplicate series was within the experimental error of the method.

The data indicate that at least 2 widely different clonal lines were isolated from the "farm" population; clone 4 appeared to be more resistant, and clone 5 more sensitive, to the viruses. In experiment 2, however, line 4 had lost its increased resistance to type 1 virus.

To establish that the differences in sensitivity noted in Table I were related to the clonal lines and not an extraneous factor, and to be more assured that the lines were of single cell origin, subclones were isolated from each of the 6 parental clonal lines. Approximately 2 months after the first clones were isolated, 4 to 6 subclones were selected from each clonal line except clone 2, from which only 2 subclones were isolated. Table II re-

TABLE II. Sensitivity of Populations of Parental Clones and Their Subclones to Types 1 and 2 Polioviruses.

Clonal line		Avg No. of plaques formed/monolayer			
		Type 1 virus		Type 2 virus	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
1	P*		37		59
	S_1†	28	36		53
	S_2	31	43		64
	S_3	20	24		39
	S_4	11	41		40
2	P	46		65	
	S_1	44		57	
3	P		22		43
	S_1	5	37	6	30
	S_2	12	44	12	41
	S_3	19	58	18	59
	S_4	15	47	15	40
4	P		41		21
	S_1	3	28	0	26
	S_2	2	32	0	26
	S_3	21	35	9	23
	S_4	2	29	0	25
5	P	41	85	223	110
	S_1	33	58	226	81
	S_2	33		223	
	S_3	46	63	232	87
	S_4	51	57	255	67
	S_5	56	75	239	67
	S_6	29‡		199‡	
6	P	46	39	41	61
	S_1	27	5	48	5
	S_2	68	55	111	70
	S_3	64	53		88
	S_4	58	58	91	69

Aliquots of same virus preparation used for each experiment; dilution used constant except for Type 2 virus, exp. 1. Parental and subclonal lines tested simultaneously.

* P = Parental clone.

† S = Subclone. Subscript refers to subclone number.

‡ Plaques smaller in size.

cords the sensitivity of the parental and subclonal lines as measured by the average number of plaques formed per monolayer of a triplicate series. Any particular parental line and its subclones were tested simultaneously with both types of virus; approximately 3 weeks intervened between the 2 experiments listed in Table II.

It is apparent that although an unexplained variability may occur on repetitive examination of a particular cell line, in general, any parental line and its subclones do not differ significantly in their sensitivity. The exception is subclone 6 of line 5 which on repeat examination was less sensitive than the parental or its sister subclonal lines, and produced a smaller plaque. It should be noted that this variant had no selective advantage in the population, since parental line 5 was maintained for more than a year with no evidence of decrease in sensitivity nor change in plaque morphology.

Line 4 and its subclones deserves comment. Three of 4 subclones were relatively resistant to the virus when first tested, but 3 weeks later this resistance was not apparent. This same sensitivity pattern had been noted with the parental line. Since no more than 3 cell passages were made in the time interval between experiments, it is unlikely that the increased sensitivity in line 4 is due to the emergence and overgrowth of a variant that is more sensitive to the viruses. Moreover, parental line 4 had already expressed its increased sensitivity at the time subclones were isolated. No detailed study of this change in sensitivity was attempted; the differences noted might have been correlated with the changes in growth characteristics which were mentioned earlier.

To minimize variables that are inherent in the test system used, all 6 parental lines and their subclones showing the highest and lowest sensitivity were tested simultaneously for their sensitivity to type 1 virus. Two different environments were used for the monolayers inoculated with the virus. One series was incubated with an agar overlay that was buffered with Tris (pH 7.2) and was not exposed to 5 per cent CO_2 in air; the other series contained an agar overlay buffered with bicarbonate which requires CO_2 to maintain pH. The results are shown in Table III. Although the average number of plaques formed per monolayer for each cell line is independent of the two environments, the plaques formed were clearer and more easily identified when a CO_2 environment was used to maintain pH. Line 5 (parental and subclone 3) is, as expected, more sensitive to the virus than any of the other lines, and its subclone 6 on repeat testing is, again, less sensitive than the parental and other subclonal lines. Line 4 has maintained its increased sensitivity.

Stability and sensitivity of lines 5 and 6 after continuous passage for 15 months. Par-

TABLE III. Simultaneous Comparison of Sensitivity of Parental and Subclonal Populations to Type 1 Poliovirus. Two different environments used for cultivation of monolayers after virus inoculation.

Clonal line		Avg No. of plaques formed/monolayer: CO_2 environment	Tris-buffered medium; no CO_2
1	P	38	29
	S_2	41	32
	S_3	33	21
2	P	33	18
	S_1	18	16
	S_2	28	24
3	P	50	43
	S_1	39	30
	S_3	60	48
4	P	37	28
	S_1	20	13
	S_3	32	21
5	P	91	102
	S_3	89	83
	S_6	31*	27*
6	P	29	29
	S_1	21	14
	S_2	45	35
Farm population		23	17

* Plaques smaller in size.

P = Parental clone; S = Subclone.

Virus preparation and dilution same as that used in Table II.

ental lines 5 and 6 were retained as the routine cell lines for the laboratory, the former because of its greater sensitivity and the latter because of its more dispersed type of growth. Line 5 produces a uniform, dark red background for plaques when exposed to neutral red, probably because the cells grow in a closely packed formation. The plaques formed are, however, rather small and have irregular edges. Plaques formed on populations of line 6 are larger and have a smooth margin, but the background color produced by cell uptake of neutral red is less intense than with line 5.

For at least a 15-month period, virus titers remained unusually constant and both cell lines maintained predictable growth rates. Despite this constancy, however, it became evident that line 5, within a year of its primary isolation, contained a small proportion of cells that appeared to grow in a looser, or more dispersed manner than the majority of cells. Subclonal lines were therefore again selected from both lines 5 and 6 and tested for their sensitivity to types 1 and 2 viruses. Two types of colonies were noted in line 5. One (subclone 7) was the closely packed type characteristic of the line; the other (subclone 8) was composed of cells that were spread out and not closely packed together. One clone of each type was isolated and subsequent passage established the difference in type of cell growth. Colonies from line 6 were uniform and characteristic; two were isolated. Populations derived from these isolates were examined for virus sensitivity. In Table IV, the average number of plaques formed on 3 to 6 monolayers per cell line and virus are listed for the parental and subclonal lines. Each experiment represents the simultaneous testing of all cell lines. Although subclone 8 (loose cell growth) of line 5 may be less sensitive than the characteristic cell type, the evidence is suggestive only since the counts in experiment 1 were too high for reliable results. Subclone 5 of line 6 appears to be less sensitive to type 2 virus than the parental line. In general, line 5 maintained its greater sensitivity to the polioviruses for more than a year despite the emergence of cells with different growth characteristics.

It is evident that variability in sensitivity exists from experiment to experiment. Within a single experiment, such factors as pH, individual serum effects, and virus dilution are

TABLE IV. Sensitivity of Populations of Parental Clonal Lines 5 and 6 and Their Subclones to Types 1 and 2 Polioviruses One Year after Primary Isolation of Parental Lines.

Clonal line	Avg* No. of plaques formed/monolayer: Type 1 virus, Exp. 1	Type 1 virus, Exp. 2	Type 1 virus, Exp. 3	Type 2 virus, Exp. 1	Type 2 virus, Exp. 2	Type 2 virus, Exp. 3
5 P	450†	137	80	58	87	92
S_7	450†	160	86	83	112	97
S_8	227	139	55	42	120	95
6 P	172	92	38	42	95	68
S_5	129	59	34	26	32	34
S_6		79	39		61	40

* Avg of 3-6 replicate platings.

† Approximate figure.

P = Parental clone. S = Subclone; subscript refers to subclone number.

All clonal lines tested simultaneously for each experiment.

Virus preparations used for Exp. 1 differed from that used for Exp. 2 and 3.

TABLE V. Influence of Size of Cell Population Seeded for Monolayers on the Sensitivity of Clonal Lines to Type 1 Poliovirus.

Clonal line		Avg No. and distribution of plaques formed in monolayers of varying seeding population sizes		
		2×10^6 cells*	1×10^6 cells*	0.5×10^6 cells*
5	P†	100 (89–104–107)	89 (106–78–84)	75 (74–76)
	S_7‡	87 (74–84–103)	101 (97–100–105)	71 (71–76–67)
	S_8	83 (93–88–74)	72 (74–62–80)	70 (75–61–74)
6	P	59 (64–59–54)	41 (36–46–42)	39 (45–35–37)

* Approximate figure. † P = Parental clonal line. ‡ S = Subclonal line.

kept relatively constant. One factor which might influence variability within a single experiment is the concentration of cells in a monolayer at the time of virus inoculation. The number of cells required to form a continuous monolayer would be expected to vary with cell size and growth pattern. To determine the importance of cell concentration, parental line 5 and its two subclones (7 and 8) and parental line 6 were tested simultaneously for their sensitivity to type 1 virus when the number of cells plated on monolayers was approximately 2 x 10^6, 1 x 10^6 and 0.5 x 10^6 cells. (One million cells was the standard concentration used.) The data are given in Table V; the average number of plaques formed per monolayer and plaque distribution in a triplicate series are shown for each cell line and concentration.

Twice the standard cell concentration does not appear to increase significantly the number of plaques formed. The slight reduction in plaque number when one-half the standard cell concentration was used was anticipated, since this concentration did not yield continuous cell monolayers. The two subclones of line 5 which differ in growth characteristics do not appear to differ significantly in their sensitivity to type 1 virus. Any difference in sensitivity between subclones 7 and 8 of line 5, if it exists, is only suggestive in this experiment, when the plating population for cell monolayers approaches the standard plating population. The greater sensitivity of line 5 does not, therefore, appear to be wholly related to its unique pattern of growth.

Summary. Propagation of HeLa cell populations from single clones isolated by the Puck technic provides cells which are more stable in growth potential and virus sensitivity than "farm" populations from which they were selected. Populations from 6 clones have been studied; 4 were of predominant cell type by microscopic examination (clones 1, 2, 3 and 6) and 2 differed from the predominant type (clones 4 and 5). Clone 5 was the most sensitive and clone 4 the most resistant to types 1 and 2 polioviruses. Although clone 5, as a generalization, maintained its characteristic morphology and sensitivity to poliovirus for 2 years, it was not possible to correlate morphology with sensitivity to the polioviruses. Selection and propagation of this most susceptible of the 6 clonal populations isolated provided a stable tool for studies of polioviruses. Since line 5 maintained its greater sensitivity for at least 2 years, a heritable factor (or factors) is probably responsible for the greater sensitivity.

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Received June 23, 1959. P.S.E.B.M., 1959, v102.

Interspecific Transformation in Hemophilus; A Possible Index of Relationship Between *H. Influenzae* and *H. aegyptius.** (25151)

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Induction of streptomycin (SM) resistance by desoxyribonucleic acid (DNA) from one SM-resistant *Hemophilus* "species" in a sensitive population of a heterologous species of this genus has been reported by 2 groups of investigators(1,2). The proportion of recipient population transformed by SM-resistance transforming factor (DNA) from donor cells of a heterologous species was only a small fraction of those in which resistance was induced by DNA from donor cells of a homologous species. Schaeffer(3) suggested that the lower frequency of heterospecific transformation is a reflection of imperfect pairing of DNA units due to structural differences in parts of the DNA molecule. A basically similar but less precise explanation has been offered for our results; the degree of reactivity of the DNA's of recipient and donor cells reflects their genetic homology, and therefore might be used as a taxonomic guide(4). When this criterion was applied to *H. influenzae, H. parainfluenzae,* and *H. suis,* members of each species appear to belong to distinct groups; *H. influenzae* and *H. parainfluenzae* are more closely related than either is to *H. suis.* This report extends these observations to strains of *H. aegyptius*(5), originally classified as Koch-Weeks bacillus; the results suggest that this organism is closely related to *H. influenzae.* The degree of reactivity of SM-resistance transforming factor, derived from *H. aegyptius* strains, with *H. influenzae* recipient populations and *vice versa* is comparable to the reactivity between types of *H. influenzae.* Pittman and Davis(5) report that the Koch-Weeks bacillus differs sufficiently from *H. influenzae* morphologically, serologically, and by its capacity to agglutinate red blood cells, to warrant separation into a new species, *H. aegyptius.* Previous views on classification of Koch-Weeks bacillus have been reviewed by Pittman.

Results. It has previously been suggested (4) that in a given recipient population of Hemophilus the ratio of number of SM-resistant cells induced by DNA from heterologous type or species to the number transformed by a homologous type or species DNA provides a numerical index of the degree of relationship of recipient and donor cells. When the donor of the DNA is of the same strain or species as the recipient population, for example, different types of *H. influenzae,* the value of this ratio approaches 1 for some types, and may be as low as 0.1 for other types. However, when the SM-resistance transforming factor is derived from a heterologous *Hemophilus* species, the value of this ratio is of a different order of magnitude, varying from 0.01 to 0.0002 depending upon recipient population used and the species under study. This influence of species source of DNA is independent of DNA concentration and competence of the recipient population, and is not due to different rates of expression of transformants or DNA extraction procedure (review, 3).

Experiments similar to those reported earlier(4) have been designed to examine the relationship of *H. aegyptius* to *H. influenzae* and *H. parainfluenzae* by determining the value of this ratio. For this purpose, DNA's containing the SM-resistance factor were prepared from 2 different strains of *H. influenzae,* three different strains of *H. aegyptius,* and 1 strain of *H. parainfluenzae.* We are indebted to Dr. Pittman for the strains classified as *H. aegyptius.* Four strains of *H. influenzae,* three of *H. aegyptius,* and 2 of *H. parainfluenzae* were used as receptor strains. In any experiment, aliquots of the same recipient population were used for comparing the proportion of transformants induced by the SM-resistance transforming factor of different origins.

* This work was supported by grants from NIH.

TABLE I. Comparison of Number of Streptomycin (SM) Resistant Cells in Which Resistance Is Induced by Transforming Factor Derived from 3 "Species" of Hemophilus—*H. influenzae*, *H. aegyptius* and *H. parainfluenzae*.

			Induced SM resistant cells/10^8 SM sensitive cells					
			Species and strain source of transforming factor (DNA_{SM})					
			H. influenzae		*H. aegyptius*			*H. parain-fluenzae*
Receptor species and strain*		Exp.	Rd	3	46	181a	15	1
H. influenzae	Rd	1	28,000		17,000			
		2	48,000		48,000	33,000	47,000	120
	Rb	1	780		510			
		2	510		180	190	260	
		3	1,880		1,030	660	1,240	23
	3	1	1,600		7,300	7,100	9,700	15
		2	3,200	11,800	13,300	8,800	13,000	25
	4	1	650		410	280	360	11
H. aegyptius	46	1	63		110			
	181a	1	780		1,300	1,200	2,500	8
		2	85	64	240	67	260	1
	15	1	250	190			830	22
		2	130	98	260	94	160	
		3	85		300	100	250	7
H. parainfluenzae	1	4			7	15	1	2,100
	7	26			15	43	23	4,500

* *H. influenzae* — Rb and Rd = Non-encapsulated variants selected from populations of Types b and d; 3 and 4 = Non-typable strains from blood and nasopharynx respectively. *H. parainfluenzae* — Isolated from nasopharynx.

The technics used for the conference of SM resistance in sensitive populations have been reported(4). For the present study, DNA extracts from cells resistant to at least 1,000 mcg/ml of streptomycin were impure preparations except for one purified DNA fraction of a strain of *H. aegyptius* (181a).† Recipient cells were exposed to DNA-containing extracts (approximately 1-2 mcg/ml of DNA) for 15 minutes and desoxyribonuclease (DNAase) then added. The treated cells, appropriately diluted, were seeded in pour plate preparations of Levinthal agar and incubated 2 hours to permit expression of the induced resistance trait before exposure to streptomycin to test for presence of resistance. Levinthal agar containing 2,000 mcg/ml of streptomycin was then layered over the pour plate preparations, and after hardening of the overlay the plates were incubated for 48 hours. Appropriate controls (desoxyribonuclease and untreated cells) were included.

Data in Table I indicate that in a given recipient population of *H. influenzae* or *H. aegyptius*, the number of cells transformed by either *H. influenzae* or *H. aegyptius* SM-resistance transforming factor is of the same order of magnitude. As shown in Table II, the hetero-homospecific transformation ratio for members of these 2 species may approach 1; the lowest value obtained is 0.2. Data from Table I and those published(4) were used to determine the ratios listed in Table II. The results obtained are therefore comparable to ratios found when comparisons are made of the number of transformants induced in a given recipient population of *H. influenzae* by DNA's derived from different type specific or non-type specific strains of *H. influenzae*((4) and Table II). On the other hand, the proportion of cells of either *H. influenzae* or *H. aegyptius* transformed to SM-resistant by DNA from *H. parainfluenzae* is of a much lower order of magnitude (Tables I and II); it is in the range expected in interspecific transformation(3,4). This is true also for strains of *H. parainfluenzae* exposed to the SM-resistance transforming factor of either *H. influenzae* or *H. aegyptius*.

Discussion. When aliquots of the same re-

† The purified product was prepared by Dr. Sheldon Greer.

TABLE II. Ratio of Number of Cells Transformed to Streptomycin (SM) Resistant by Heterologous "Species" DNA_{SM} to Number Transformed by Homologous Species DNA_{SM}. Range of ratio for homologous species transformation included for comparison.

Recipient species and strain		Hemophilus species source of transforming factor (DNA_{SM})		
		H. influenzae	*H. aegyptius*	*H. parainfluenzae*
H. influenzae	Rd	(.3 to 1)*	.6 to 1	.003 (.002 to .004)*
	Rb	(.2 to .8)*	.4 to .7	.012 (.007 to .008)*
	3	(.1 to >1)*	.7 to >1	.002 to .009
	4	(.3 to .6)*	.4 to .6	.017 (.016 to .02)*
H. aegyptius	46	.6		
	181a	.3 to 1	.3 to 1	.003 to .006
	15	.2 to 1	.3 to .8	.02 to .07
H. parainfluenzae	1	.002 (.003–.004)*	.003 to .007	(.1 to .5)*
	7	.006	.003 to .009	(.3 to 1)*

* Figures in parentheses obtained from published data(4).

cipient population of *H. influenzae* or *H. aegyptius* are exposed simultaneously to DNA's derived from streptomycin (SM)-resistant cells of the same strain or from different *H. influenzae* and *H. aegyptius* strains, the proportion of cells in which SM-resistance is induced is of the same order of magnitude. The results obtained are comparable to those found in intraspecific transformation(4). On the other hand, the proportion of cells of either *H. influenzae* or *H. aegyptius* transformed to SM-resistant by the DNA from *H. parainfluenzae* is in the range expected in interspecific transformation (4).

Summary. On the premise that the degree of reactivity of donor DNA with the heredity determinants of the recipient cell is a reflection of the degree of homology of the genome of donor and recipient cells, it has been proposed that transformation may serve as a valid criterion for bacterial taxonomy. If the use of a single genetic marker, SM-resistance, provides a valid basis for comparison, *H. aegyptius* may be a member of the *H. influenzae* group.

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Received June 23, 1959. P.S.E.B.M., 1959, v102.

Effect of Certain Tranquilizers on The Reticulo Endothelial System. (25152)

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Several investigators observed that administration of reserpine or chlorpromazine lowered the resistance of patients to infectious diseases(7,9). Grosz and Norton(4) reported that chlorpromazine increased the susceptibility of mice to infection with *Salmonella enteritidis.* Meier *et al.*(8) showed that chlorpromazine depressed the phagocytic action of the reticulo endothelial system (RES) whereas mepazine (N-methyl-piperidyl-1,3-methyl phenothiazine) did not have this action. It was of interest to study the effect of several tranquilizers on the RES in greater detail and to carry out experiments relating to their mode of action.

Methods. Measurement of RES activity was accomplished by determining blood clearance of carbon particles (india ink, Gunther-Wagner, Hanover, Germany C11/1431A) as outlined by Heller *et al*(5). Average particle

TABLE I. Effect of Tranquilizers on Clearance Rate of Carbon Particles in Blood of Mice. T/2 is time in minutes needed to reduce initial carbon level to one half.

Drug	Dose, mg/kg	Control		Drug		T/2 as % of control	P value
		No. of animals	T/2 ± S.E.	No. of animals	T/2 ± S.E.		
Chlorpromazine	5	5	22.3 ± 3.6	5	19.5 ± 1.8	87.4	.5
	20	9	21.1 ± 2.1	8	29.6 ± 2.5	140.3	.02
Hydroxyzine	50	5	22.3 ± 3.6	6	19.0 ± 1.9	85.4	.4
	100	9	21.1 ± 2.1	9	33.3 ± 2.8	152.6	.001
Meprobamate	"	6	21.3 ± 2.0	6	23.7 ± 2.7	111.1	.5
	200	5	22.3 ± 3.6	5	" ± 2.9	106.3	.8
Phenaglycodol	100	9	21.1 ± 2.1	9	27.3 ± 1.6	129.2	.02
	200	5	22.3 ± 3.6	5	46.0 ± 2.9	206.1	.001
Reserpine	1	5	" ± "	6	23.7 ± 3.2	106.3	.8
	10	9	21.1 ± 2.1	8	46.1 ± 6.4	218.4	.001

size in this suspension is about 200 A. Male CF1 mice weighing 20 to 22 g were used as experimental animals. Tranquilizers were given intraperitoneally. Chlorpromazine, hydroxyzine and pentobarbital were dissolved in saline. Meprobamate was given as a supersaturated solution in saline. The volume given was 0.5 ml/20 g mouse. Reserpine and phenaglycodol were dissolved in minimum amount of propylene glycol. Most drugs were injected at 2 different dose levels. Carbon suspension was injected intravenously 24 hours after administration of drugs in a dose of 200 mg/kg in a 0.2 ml volume/20 g mouse. Blood samples were obtained from the tail 8, 15 and 22 minutes after administration of carbon suspension. Each 0.005 ml blood sample was diluted with 0.6 ml of 0.01 M sodium carbonate and the optical density determined on Bausch and Lomb spectrophotometer at 600 mμ. At least 6 animals were used for each determination. Density values for each experimental animal were plotted on semi-logarithmic paper and the half life of carbon clearance determined. Average half time and its standard error were then computed. A control group receiving saline was performed with each experiment. Adrenalectomy was carried out under ether by the dorsal approach. Animals were given one week to recover and received a 1% NaCl solution in place of drinking water.

Results. Chlorpromazine, hydroxyzine and reserpine did not significantly affect the clearing rate at low doses but markedly slowed clearing of carbon particles from the blood stream in high doses. Phenaglycodol had a slight but significant effect at lower dose and produced a striking delay of clearing at higher dose. The only tranquilizer that did not affect the clearing rate was meprobamate. These results are shown in Table I.

It is known that chlorpromazine stimulates release of corticotropin from the anterior pituitary(3) and that corticosteroids inhibit clearing of carbon particles by the RES(6,2). From these facts it appeared possible that tranquilizers which delay clearing, do so by stimulating secretion of corticosteroids. To investigate this possibility clearing rates in adrenalectomized animals were studied.

Adrenalectomized animals did not tolerate the large doses of chlorpromazine and reserpine that had to be given to retard clearing. The animals died within 24 hours after administration of the drugs. Adrenalectomized animals receiving hydroxyzine tolerated the drug and could be used for this study. Results of an experiment are shown in Table II. Adrenalectomy does not affect clearing, as previously reported(1). Hydroxyzine in untreated animals delayed clearing but clearing was unaffected in drug-treated adrenalectomized animals. Sham-operated animals treated with hydroxyzine showed a significant delay in clearing rate.

Discussion. The results here reported indicate that certain tranquilizers when given in large doses have a depressant action on the reticulo endothelial system. This is apparently due to a stimulant effect of these drugs on secretion of corticosteroid hormones by the adrenal gland. It is possible that the increased

TABLE II. Effect of Adrenalectomy on Carbon Clearance of Mice Treated with Hydroxyzine.

Treatment	Dose, mg/kg	No. of animals	T/2 ± S.E.	P value
A. Normal, saline		9	27.0 ± 2.6	
B. Adrenalectomy, saline		9	24.9 ± 2.5	.6
C. Normal, hydroxyzine	100	10	59.6 ±10.7	.01
D. Adrenalectomy, hydroxyzine	"	9	25.1 ± 2.2	.6
E. Sham operation, hydroxyzine	"	9	39.5 ± 3.5	.01

susceptibility to infections as well as certain other side effects observed after administration of large doses of certain tranquilizers, may be due to an excess of steroid hormones in the body. However, the data presented here indicate that tranquilizers given in the usual clinical doses would not be likely to affect the reticulo endothelial system.

Several tranquilizers when given in large doses produced a marked depression of the central nervous system, manifested by deep sedation and loss of the righting reflex. The possibility that this central nervous system depression may affect phagocytic activity of the reticulo endothelial system was tested by determining clearance rates in animals treated with pentobarbital 50 mg/kg and amobarbital 140 mg/kg, These hypnotics which produced deep central nervous system depression with loss of righting reflex did not inhibit phagocytic activity of the reticulo endothelial system.

Summary. Administration of excessive doses of certain tranquilizers decreased the phagocytic activity of the reticulo endothelial system. This decrease of phagocytic activity did not occur in adrenalectomized animals given hydroxyzine. The significance of this finding is discussed in relation to the strong RES inhibition produced by corticosteroids (6,2).

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Received June 25, 1959. P.S.E.B.M., 1959, v102.

Serum Lipid Analyses in Rats Fed Natural and Hydrogenated Cottonseed Oil with Cholesterol and Cholate.* (25153)

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In a recent study we recorded some measurements of rat blood lipid concentrations when dietary cholesterol was present or absent in rations with varying degrees of saturation of vegetable fats(1). The results indicated that serum cholesterol is raised by increasing saturation of dietary lipids and that dietary cholesterol augments this effect for more saturated fats. In this experiment, we extended our observations to include lipoproteins and fatty acid compositions of serum cholesterol esters when saturated (hydroge-

* This investigation supported by grant from Nat. Inst. Health, U.S.P.H.S.

nated) and unsaturated vegetable fats are fed to rats with or without sodium cholate and dietary cholesterol.

Methods and materials. Animals. Sixty, 3-month-old albino male Sprague-Dawley rats were marked, weighed and bled for serum lipid determinations. Animals had average weight of 344 g (310-399 g) and had been maintained on Purina Laboratory chow. They were divided into 6 groups comparable with respect to distribution of initial weight and serum lipid concentrations. All rats were kept in a thermostatically controlled room (76°F), in large wire-bottomed cages with 10 rats/cage. *Diet.* A liquid ration, described by Moskowitz, *et al.*(2) of 50% fat, 28% carbohydrate, 5% protein, and 1% choline (on dry weight basis) and containing adequate minerals, vitamins, and roughage was used. Three diets were made with each of 2 fats: cottonseed oil, iodine number (I# 107) and hydrogenated cottonseed oil (I # 78) and each was fed to 10 rats. Detailed analyses of these fats have been presented(1). One diet with each fat was fed with 0.4% sodium cholate, or with 0.4% sodium cholate and 1% cholesterol, or without these additions. Diets were prepared and fed by stomach tube as previously described(1). *Serum lipids.* Methods described earlier were used for serum cholesterol and lipid phosphorus(1). Lipoproteins were analyzed by a standard method of paper electrophoresis(3). Total serum cholesterol was separated into free and esterified parts by alumina column chromatography using a modification of Kerr and Bauld's technic(4). Silicic acid column chromatography yielded pure cholesterol ester fractions using method of Riemenschneider(5). Cholesterol esters were then saponified and methylated using methanol and sulphuric acid. Methylated fatty acids obtained from cholesterol-ester serum fractions were analyzed by gas chromatography for chain length and unsaturation as described by James(6). *Histopathological technic.* All animals were autopsied when they died or when sacrificed at end of experiments. Rats were exsanguinated from abdominal aorta under ether anesthesia. Organs were examined grossly and tissue samples of heart (transverse and frontal blocks) as well as all other organs were fixed in neutral formalin-saline. Opened aortas were fixed flat in buffered formalin-saline, and stained for gross neutral fat with Sudan IV. Duplicate blocks were taken of aorta (both ends) for frozen sections stained for neutral fat with Sudan IV. Other tissue blocks were embedded in paraffin, cut at 6 μ, and stained with hematoxylin-eosin.

Results. Body and liver weights. All groups gained weight during experiment. There were identical slight weight increases in groups fed the natural oil (I# 107) with cholate or cholate and cholesterol. However, when cholate was aded to the hydrogenated fat (I# 78) weight increase was markedly diminished. This decrease in weight gain was accentuated when cholate and cholesterol were added together. For unsaturated dietary oil, added cholate caused increased liver weights. Combination with dietary cholate and cholesterol further increased liver weight. For saturated dietary fat, only the combination with both cholesterol and cholate resulted in increased liver weight and then not to levels seen with these additions to unsaturated oil.

Histopathology. Study of the histological sections in this experiment revealed no atherosclerotic lesions in coronary arteries, aortic or mitral valves or proximal or distal aorta.

Serum lipid determinations. Fig. 1 shows results of serum total cholesterol and phospholipid determinations. For all groups serum cholesterol rose to a peak at 9 or 11 weeks. There was a fall in values after achieving this peak. For both unsaturated and saturated lipid, added dietary cholate did not alter serum cholesterol levels from those achieved by the oil fed without additions. However, when both dietary cholate and cholesterol were added together, serum cholesterol rose. This effect was far greater for saturated fat. Phospholipid values and C/PL† ratios were closely parallel to serum cholesterol values. Increasing saturation from I# 107 to I# 78 or adding 0.4% cholate to either fat did not increase the C/PL ratio. However, the ratio was slightly elevated when both cholate and cholesterol

† Serum cholesterol/phospholipid ratios.

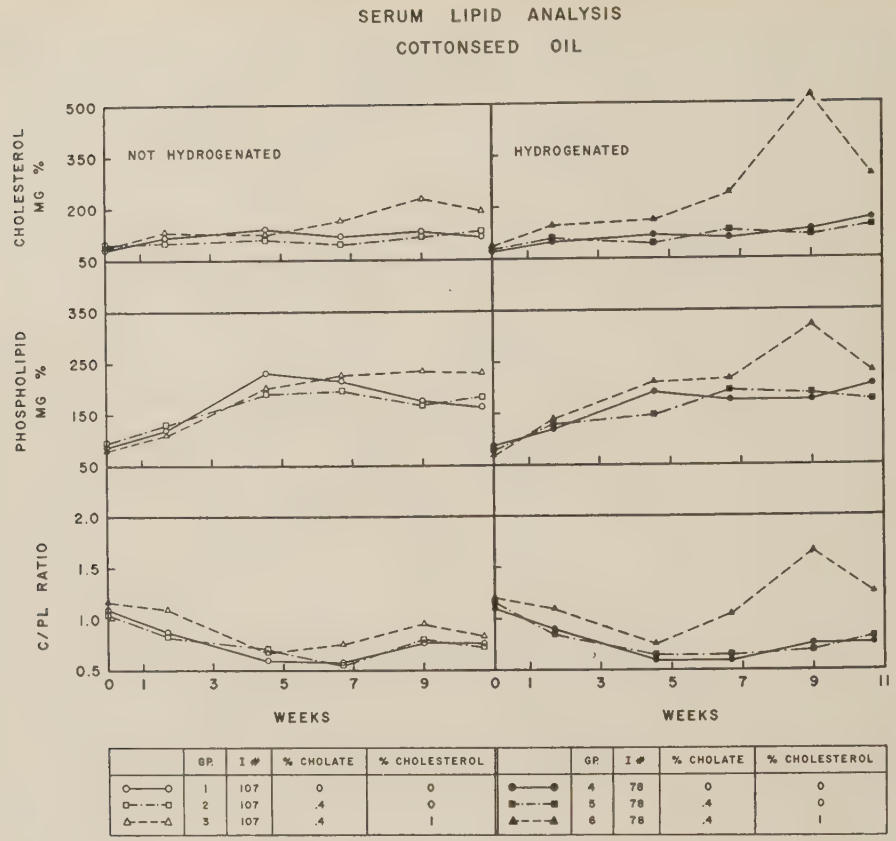


FIG. 1. Mean serum lipid analyses.

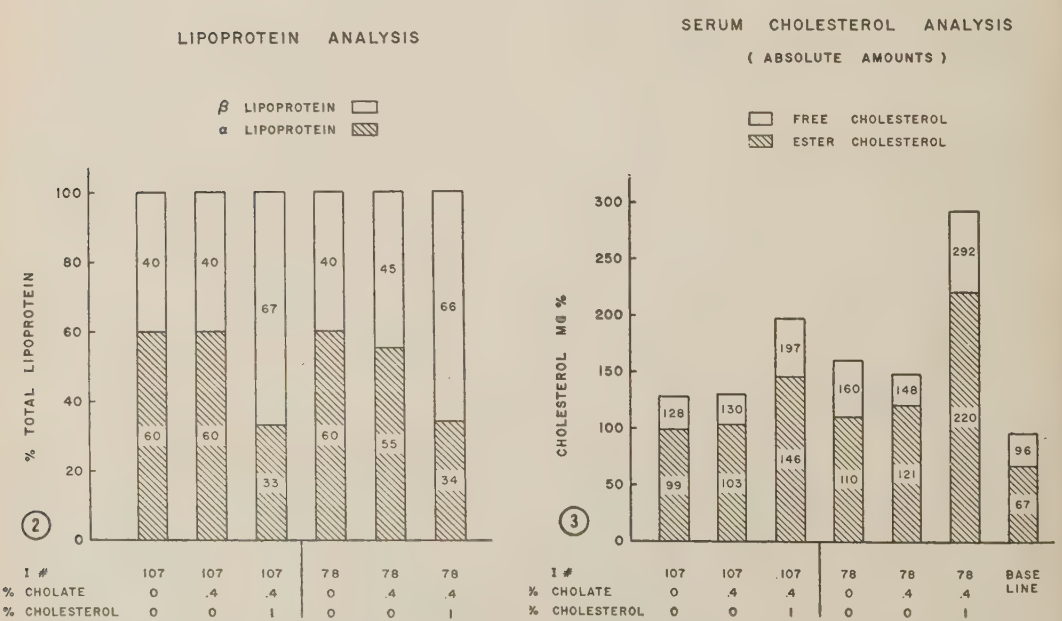
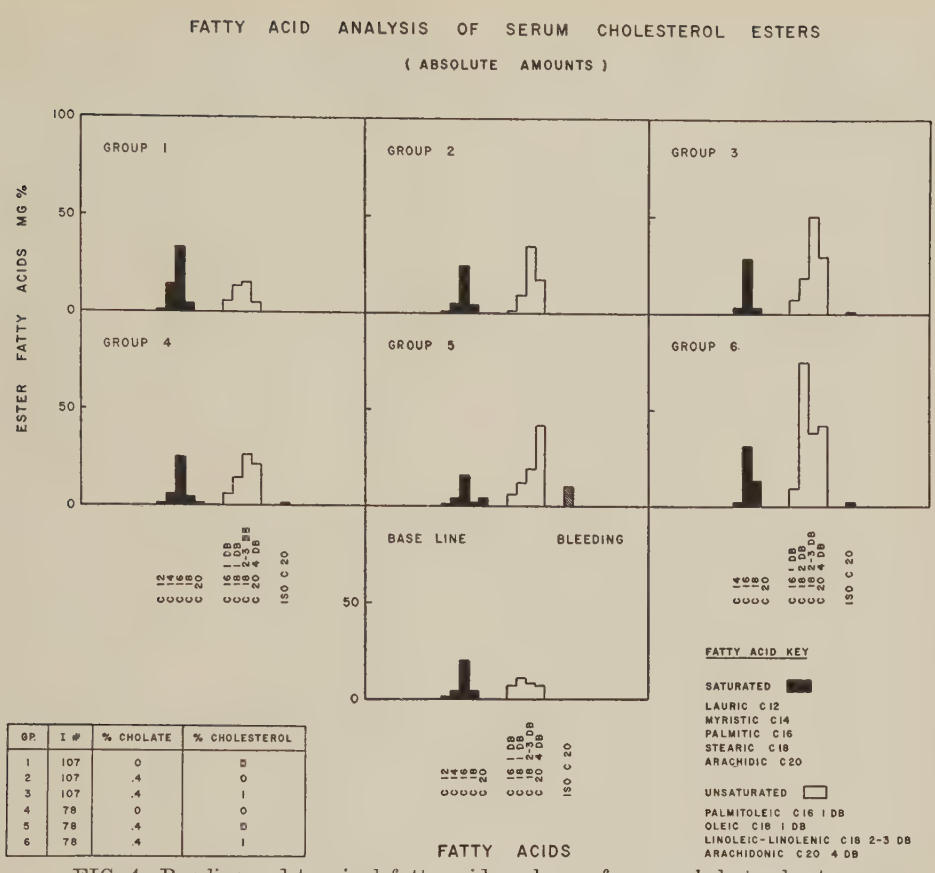


FIG. 2. Terminal lipoprotein analyses of pooled serum (relative values only).
FIG. 3. Pooled serum cholesterol in absolute amounts. Top figure is total serum cholesterol. Bottom figure is amount of esterified portion.



GP.	I #	% CHOLATE	% CHOLESTEROL
1	107	0	0
2	107	.4	0
3	107	.4	1
4	78	0	0
5	78	.4	0
6	78	.4	1

FIG. 4. Baseline and terminal fatty acid analyses of serum cholesterol esters.

were added to natural cottonseed oil (I# 107) and greatly raised when these additions were made to saturated fat (I# 78). The latter was the only dietary group which maintained C/PL elevation at end of experiment.

Fig. 2 shows relative values for Alpha and Beta lipoprotein (LP) analyses done at termination of experiment. Alpha LP predominated. For both lipids, when combination of dietary cholate and cholesterol was added, the previous relation reversed, and Beta LP became dominant fraction. In work previously published(1) we found that only extremely hydrogenated cottonseed oil (I# 43) fed with cholesterol would cause Alpha to Beta LP ratio to reverse and Beta LP to become dominant. In contrast to this result, combination of dietary cholate and cholesterol permits reversal of Alpha to Beta LP ratio with moderate or no hydrogenation of dietary fat.

Fig. 3 shows fractionation of terminal serum cholesterol into free and esterified portions. High fat feeding for both unhydrogenated and hydrogenated fat caused an increase in serum esterified cholesterol. Increased saturation of hydrogenated lipid slightly increased ester content. Added cholate with both fats further increased ester values; more so for saturated fat.

Fig. 4 shows cholesterol ester fatty acid analyses in mg %. For both unhydrogenated and hydrogenated fats with addition of cholate or cholate with cholesterol, there was a stepwise tendency for esterification to occur more with unsaturated fatty acids than with saturated acids. Considering saturated serum esters, with either dietary lipid, C-14 myristic acid declined with added cholate or cholate and cholesterol. For all groups C-16 palmitic acid was the saturated ester acid in greatest amount. Addition of cholate alone to both fats caused a decrease in C-16 saturated acid

but added cholate with cholesterol raised values to previous levels. This effect was more pronounced with saturated dietary fat. Of the unsaturated acids, C-18 oleic acid was altered in a way similar to C-16 palmitic acid, with some decline with added cholate and restoration with cholate and cholesterol. In the groups fed natural oil (I# 107) linoleic-linolenic acids were the most abundant unsaturated ester acids. Added cholate increased these acids. There was another increase when both cholate and cholesterol were fed. For the group fed hydrogenated lipid alone, linoleic-linolenic was most abundant of unsaturated esters. When cholate was added, unlike its unhydrogenated counterpart, esterification pattern shifted so that arachidonic acid was most abundant unsaturated acid. When cholesterol with cholate was fed, esterification again shifted and now was greatest with oleic acid. This was the group with highest values for serum cholesterol, phospholipid, and C/PL.

Discussion. In view of results of Hegsted *et al.*(15) under experimental conditions similar to ours, we expected to observe cardiovascular sudanophilia in our animals. However, microscopic lesions were not seen, possibly because of differences in the detailed design of the experiments. For example we employed whole egg as dietary protein while they used casein. Furthermore their dietary choline and fat levels were much lower. The fatty acid composition of esterified serum cholesterol is of interest in view of suggestions made about effect of saturated versus unsaturated acids on atheromatous deposits. It has been stated that predominant saturation of dietary lipid may cause a prepondernace of saturated cholesterol esters in blood and tissues(8). These sterols esterified with saturated acids have higher melting range, are less soluble, and possibly less compatible in blood. Of more importance, they are turned over more slowly in tissues than are unsaturated essential fatty acid esters(9). Thus saturated sterol esters may be relatively inert and have greater tendency to accumulate in artery walls. There is evidence that sterol esterified oleic acid is found in greater abundance in atheromatous plaques than in serum(10). Possibly then, oleic acid is a partially unsaturated acid which when incorporated in cholesterol esters is metabolically equivalent to saturated acids and thus more likely to accumulate in vessel walls. In this experiment oleic acid rose markedly in the group which had highest serum cholesterol, Beta lipoprotein and C/PL. James described an increase of oleic acid in serum esterified cholesterol and triglycerides of coronary patients(11) thus supporting the idea that a fault in lipid metabolism involving oleic acid contributes to coronary artery disease. Our experiment shows that dietary fat saturation and additions of cholesterol with cholate or cholate alone influence esterification of serum cholesterol. There is evidence that this is true for other serum lipids and for liver and depot fat(12,13,14).

Our results show that not only serum cholesterol level but also qualitative and quantitative composition of cholesterol esters, as influenced by dietary factors, is likely to be significant in understanding elevation of serum lipids and deposition of lipids in arteries.

Summary. 1. Dietary cholate with cholesterol added to natural and hydrogenated cottonseed oil raised serum cholesterol and phospholipid. This effect was augmented with saturated fat. Dietary lipid saturation or cholate alone had no effect on serum cholesterol. 2. C/PL ratio was elevated only when both dietary cholate and cholesterol were added to hydrogenated fat. 3. In the rat, highly saturated fat (I# 43) fed with cholesterol reverses the Alpha to Beta LP ratio with Beta LP becoming dominant. Combination of dietary cholate with cholesterol permits reversal of Alpha to Beta LP with moderate (I# 78) or no hydrogenation (I# 107) of dietary fat. 4. Serum cholesterol ester fatty acid composition changes with greater saturation of dietary fat and added cholate and cholesterol. High oleic acid levels were noted in serum cholesterol ester fraction of rats fed saturated fat (I# 78) with cholate and cholesterol.

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Received June 25, 1959. P.S.E.B.M., 1959, v102.

Quantitative Determination of C^{14}-Tyrosine Absorption in Melanated and Non-Melanated Mouse Tissues.* (25154)

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Historically the study of tyrosine metabolism is coincident with the study of genetically controlled alcaptonuria, a condition characterized by excretion of a tyrosine derivative, homogentisic acid(1). Many investigations as reviewed by Knox(2), and Meister(3), have been carried out on pathways of tyrosine metabolism. More recently, radioactively labelled tyrosine has been employed, with excellent results, in the study of the tyrosine metabolism mechanism(4,5,6). The role of tyrosine in melanosarcoma formation has also received considerable attention(7,8), and in addition the selective absorption of this tagged amino acid into various tissue proteins has been established(9). It is our purpose to study selective absorption of C^{14}-labelled tyrosine by both melanotic and non-melanotic tissues of the mouse by means of quantitative uptake determinations. S91 melanoma tissue is also included in the analyses. Since the method utilized provides a uniform assay procedure and geometry, it is felt that many technical quantitative problems are eliminated; *e.g.,* variation in self-absorption between dissimilar tissue samples.

* This work supported in part by research grant from Am. Cancer Soc. (Mass. Division).

Materials and methods. A. The mice used were of the dba/1 Jackson strain subsequently inoculated with Cloudman S91 melanoma. All animals were of approximately the same age and weight (6 mo., 15 g). B. *Radioisotope administration.* Five microcuries of dl-tyrosine-2-C^{14} (Tracerlab, Inc.) were prepared and administered according to standard procedure(7) to each animal, 5 weeks after tumor inoculation. Twelve animals were used during the investigation. C. *Tissue preparation.* Each animal was sacrificed 7 days after radioisotope injection with immediate removal of melanoma, liver, kidney, brain, adrenal, thyroid, and combined skin and hair tissue samples. Each sample was placed in a small pyrex tube, quick frozen by immersion into acetone-dry ice mixture, and stored at 0-4°C until time of analysis. D. *Tissue analysis.* To eliminate variabilities encountered by use of wet weights as measurement of tissue sample and to put all tissues used on a comparable and accurate assay basis, a modification of the Van Slyke-Folch(10,11) tissue combustion procedure for determination of tissue carbon content was employed. The manometrically determined quantity of CO_2 produced, was introduced into barium hydroxide solution

TABLE I. Selective Absorption of C^{14}-Tyrosine/mg Carbon in Melanated and Non-melanated Mouse Tissues.

			Mean (X) values			
			I	II	III	IV
Tissue	No. of animals	No. of samples	mg carbon ($PCO_2 \times F^*$)	Activity ($N_s - N_b$)	$\left(\frac{\text{II}}{\text{I} \times 200^\dagger}\right)$ mμC/mg C	$\left(\frac{\text{III} \times 100 \times 1000}{5^\ddagger}\right)$ % tyrosine $\times 10^{-3}$ absorbed/mg C
Melanoma	12	24	2.433	223	.4583	9.17
Kidney	"	"	2.913	191	.3278	6.56
Adrenal	"	12	1.015	49	.2413	4.83
Liver	"	24	3.267	135	.2066	4.13
Brain	"	"	2.706	94	.1737	3.47
Thyroid	11	11	.583	13	.1114	2.23
Skin & hair	12	23	3.792	75	.0989	1.98

* Correction factor (1.027) for Van Slyke combustion apparatus used.
† Tyrosine standard: 200 cpm ($N_s - N_b$; sample cpm minus background cpm) = 1 mμC activity.
‡ Total tyrosine activity inj./animal = 5 μC.

and precipitated as barium carbonate. By this method, a precipitated, $BaCO_3$ yield, representing the carbon content of small tissue samples (20-30 mg) could be assayed for its radioactivity content. By similar procedure (12), a series of C^{14}-tyrosine micro-combustion standards was prepared, to relate the obtained number of counts/minute in any tissue analysis to the actual fraction of millimicrocuries of C^{14}-tyrosine present (absorbed) within the sample. Due to the infinitesimally thin $BaCO_3$ layer produced from any sample of tissue, it was unnecessary to make self-absorption corrections between samples. Radioassay procedures were carried out by triplicate planchet analyses of dried $BaCO_3$ samples wtih a windowless gas flow proportional counter and pre-set time scaler. After interpolating the counts/min/mg of carbon (as manometrically determined), specific activity was expressed as millimicrocuries (or fraction thereof) of C^{14}-tyrosine/mg carbon of the tissue sample. The "t" distribution test(13) was used to determine statistical significance of the data. A P value of 0.05 was chosen as the level of significance (95% confidence interval).

Results. Selective absorption of C^{14}-tyrosine in tissues assayed is presented on a comparative basis in Table I. The order of tyrosine uptake by the tissues (/mg carbon as measured manometrically) was melanoma, kidney, adrenal, liver, brain, thyroid, and skin and hair. Despite some individual variations noted in composite assay of any single tissue, significant differences in uptake (Table II) exist between all tissues with the exception of comparisons between: *a*) adrenal tissue and kidney, liver, brain, or thyroid; *b*) thyroid and skin and hair; *c*) liver and brain. Lack of significant difference between these tissues may indicate a relatively equal selective absorption of the amino acid. It is important to note that melanoma tissue selectively ab-

TABLE II. Statistical Evaluation of Selective Absorption of C^{14}-Tyrosine in Melanated and Non-melanated Mouse Tissues.

Tissue	Cases		Melanoma	Kidney	Adrenal	Liver	Brain	Thyroid	Skin & hair
						"t" values			
Melanoma	24			4.03	3.38	6.63	7.59	5.84	9.21
Kidney	"		<.05		1.83	5.15	6.97	6.05	3.20
Adrenal	12		"	>.05; <.10		.82	1.59	1.93	3.24
Liver	24		"	<.05	>.05		1.85	3.23	5.97
Brain	"		"	"	"	>.05		2.33	4.69
Thyroid	11	"P" values	"	"	>.05; <.10	"	<.05		.46
Skin & hair	23		"	"	<.05	"	"	>.05	

sorbed a significantly greater amount of tyrosine than did any of the other tissues studied. The high order of activity noted in kidney tissue may be attributed to biological turnover of tyrosine, with the continual elimination of tyrosine's metabolic by-products by way of the excretory system. Although urinary activity was noted at its highest during the first 72 hours after radioisotope injection, it maintained a much more reduced level during remaining course of experiment.

The high level of C^{14}-tyrosine activity observed in melanoma tissue supports preliminary results(7,8), where conclusive quantitative evidence may be lacking and considerable uptake variation was noted between animals. Of all tissues studied, values obtained for adrenal and thyroid tissues showed the greatest variability. Similar high levels of activity have been reported for these tissues(7), but a great margin of error (25%) was indicated. This was attributed to tissue weight error and low counter efficiency. In our investigation, measurements were made on a standardized basis (determination of carbon content in each tissue sample), thus eliminating this difficulty. With this procedure, the primary source of error is the combustion of extraneous fatty tissue adhering to the desired tissue sample. Such error, which is readily eliminated by careful preparation of the tissue sample, yields a high carbon determination with a correspondingly low radioassay activity/mg of carbon.

Work in progress indicates a much lower kidney activity at 5 weeks after radioisotope injection, thus supporting previous results(8) which indicate the biological turnover rate of tyrosine. It is assumed that the present activity value obtained for kidney tissue is not due entirely to incorporation of tyrosine into tissue proteins, but to elimination by excretion of d-tyrosine and intermediary breakdown products of l-tyrosine metabolism.

The application of the carbon combustion method used demonstrates it as highly superior to other methods. Reproducibility of results, as demonstrated in both analyses of tyrosine standards (actual carbon content *vs.* theoretical carbon content) and most tissue samples, make the technic feasible. The radioassay of C^{14} (as $C^{14}O_2$) precipitated as $BaCO_3$ affords a greater reproducibility than previous technics (tissue slice preparation, etc.) where self-absorption of C^{14} by tissues is a major factor. It is suggested that the present procedure can be employed to further elucidate, on an accurate quantitative basis, numerous metabolic relationships other than that of tyrosine metabolism either *in vivo* or *in vitro*.

Summary. Five microcuries of dl-tyrosine-2-C^{14} were administered to S91 melanoma-bearing mice (dba/1 strain) 7 days prior to time of sacrifice. Melanated and non-melanated tissues were quantitatively assayed for selective absorption of labelled tyrosine and/or its derivatives. The order of uptake (/mg tissue carbon as measured manometrically) was highest in melanoma tissue and progressively lower in kidney, adrenal, liver, brain, thyroid, and skin and hair tissues. The combined technic of tissue combustion and precipitation of CO_2 as barium carbonate provides an extremely sensitive and reproducible activity measurement of labelled tyrosine uptake.

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Received June 26, 1959. P.S.E.B.M., 1959, v102.

Influence of Sex Hormones on Total Serum Copper.* (25155)

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Previous studies(1,2) suggested that total serum copper level increases in pregnant women. Estrogen therapy(3) has shown an increase in total serum copper in certain diseases. The pathway and relationship between hormones and serum copper is unknown, and it is not clear what part release of hormones plays in mobilization of serum copper. We were particularly interested in studying the influence of parenterally administered androgenic and estrogenic hormones on total serum copper in geriatric patients. We found that 200 geriatric patients showed a mean value of 99 ± 15 μg/100 ml as compared with a mean value of 110 ± 13 μg/100 ml serum as reported by several other authors for middle aged subjects(6).

Method. Eighteen female and 21 male subjects were selected, ranging from 66 to 95 years of age. All subjects were ambulatory, with no acute or debilitating disease and with no hypercupremia. The subjects were divided into 2 groups. Group A was composed of 5 females who received 2 mg of estradiol benzoate† 3 times a week for 7 weeks, and 5 females and 11 males who received the same medication for 4 weeks. Group B was composed of 8 females and 10 males who received 25 mg testosterone propionate† twice weekly for 10 weeks. The subjects received no other drug therapy during entire period of study. Duplicate total serum copper analyses were made prior to, and at termination of, administration of hormones, and 8 weeks after discontinuance of therapy. Blood for analysis was secured with necessary precautions to avoid contamination. All patients were in basal state. Total serum copper was determined colorimetrically, using the method of Greenleaf(4) with modification by one of the authors (NCJ).

Results. Table I shows mean total serum copper in the control period, at the end of therapy, and 8 weeks after discontinuance. In all instances there was an increase in total serum copper which was significant at the 1% level in all groups except for males in Group B on androgen, who showed an increase which was significant at the 2% level. Total serum copper returned to, or near, its preadministration value 8 weeks after hormone therapy was terminated. No correlation could be found between amount of increase in serum copper values and intensity of changes in vaginal smears after sex hormone administration.

Discussion. Several investigators observed (1,2,3) an increase in total serum copper in pregnancy, menstrual cycle, and estrogen therapy. Investigations have also shown that day-to-day changes in serum copper level are

TABLE I. Total Serum Copper Levels before and after Sex Hormone Therapy (μg/100 ml).

	Duration of therapy (wk)	Control period	End of therapy	8 wk after discontinuance
Group A (2 mg estradiol benzoate 3 times weekly)				
5 females	4	111 ± 18	137 ± 15	108 ± 12
5 "	7	110.6 ± 22	154 ± 30	99 ± 15
11 males	4	104 ± 18	135 ± 19	99 ± 20
Group B (25 mg testosterone propionate twice weekly)				
8 females	10	114 ± 15	153 ± 17	113 ± 13
10 males	10	106 ± 18	137 ± 18	105 ± 18

All values expressed as mean ± S.D.

* Supported by grants from Squibb Inst. for Med. Research and Gerontological Research Fn.

† Generously supplied by Schering Corp.

not significant (p greater than 20%) and week-to-week changes are very small(1,5,6). According to these reports and our own experience, we cannot consider the changes observed in our study as normal variations in serum copper level, as all individuals showed definite increases. Since both androgen and estrogen increased serum copper content in both male and female, there must exist some relationship between sex hormone level in the body and serum copper content.

Summary. 1. Administration of testosterone propionate or estradiol benzoate to healthy elderly males and post-menopausal women increased significantly their serum copper content. 2. Eight weeks after discontinuance of androgen or estrogen administration, serum copper values dropped back to, or very close to, preadministration levels.

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Received June 29, 1959. P.S.E.B.M., 1959, v102.

Transplantation of Polyoma Virus Induced Tumor in the Hamster. (25156)

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Polyoma virus was isolated from cell-free extracts of parotid gland tumors which had been produced by inoculation of newborn mice with similar extracts from leukemia #60 or spontaneous AKR leukemias(1). The demonstration that this agent would multiply in monkey kidney tissue culture(2), cause cytopathic lesions in mouse embryo tissue culture(3), and hemagglutination of guinea pig rbc(4) have made experiments with this virus practical. The virus is capable of producing a variety of tumor types when inoculated into newborn mice(5) and hamsters(3) but no evidence of tumor production or infection in adults except production of antiviral antibodies. This is a report of the successful transplantation in adult Syrian hamsters of a sarcoma produced by inoculation of a newborn hamster with polyoma virus, cultivation of the transplantable tumor in tissue culture and the relationship of virus and viral antibody to its growth.

Methods. (a) *Transplantation.* Healthy appearing areas of tumors were removed and minced with scissors in Eagle's tissue culture medium to give a heavy suspension of tumor fragments and 0.2 ml inoculated subcutaneously into the right scapular area of either newborn or adult hamsters. Animals were observed twice weekly for signs of tumor development. (b) *Demonstration of virus.* Either mouse embryo tissue cultures were inoculated and observed for cytopathic effect and development of hemagglutinins (HA) or 5 adult mice were inoculated intraperitoneally, bled 3 weeks later and their serum tested for hemagglutinin inhibiting antibodies(HI). (c) *HA and HI tests.* The technic and criteria described by Eddy, *et al.*(4) were used. (d) *Tissue culture.* Standard procedures of trypsinization and planting in T30 flasks were used with 10% calf serum Eagle's medium for growth and 2% horse serum Eagle's for maintenance. Maintenance medium was changed twice weekly.

Results. (a) *Transplantation series.* Hamster C474, kindly supplied by Dr. Bernice Eddy, had a subcutaneous sarcoma of approximately 5 weeks' duration, measuring 3 x 6 inches. Its serum had an HI titer of 640.

TABLE I. Transplantation History of C474 Tumor and Hemagglutination Inhibition (HI) Antibody Levels in Adult Hamsters.

Transfer #	Hamster #	Day Palpable	Day Transfer	HI antibodies Day	HI antibodies Titer*
1	2099	41	54		
2	1957	10	17	17	40
	1962	10		28	<40
	Litter	10	15	28	40
3	25	11	14	0	<40
				14	"
	8	11		0	40
				35	<40
	2041†	12		16	160
4	2030	10	20	0	<40
				20	"
	2027	10		0	<20
				20	"
				41	160
5	2031	8	20	20	<40
	2035	8			
6	1904	7	26	0	<40
				26	"
	1908	7		36	20
7	1901	7	16	16	<20
	1915	7		29	"
8	2210	6			
	2215	6			

* Reciprocal of highest dilution giving inhibition.

† Received transfer from P_2 suckling tumor.

This hamster had been inoculated at 5 days of age with mouse embryo tissue culture propagated polyoma virus and developed a subcutaneous tumor 7½ months later. A minced suspension of a portion of this tumor was inoculated IP into adult hamster 2099 with development of a subcutaneous tumor at site of inoculation 41 days later. This tumor has been transplanted 8 times (Table I). Another adult hamster (2098) also received a suspension of cells from original tumor by IP route and subsequently inoculated with frozen tumor suspension twice weekly for 8 doses. A large intraperitoneal tumor was first noticed in this hamster 84 days from first inoculation at which time its serum had an HI titer of 40. A portion of the original C474 tumor was emulsified, filtered through an ultra-fine sintered glass filter and inoculated subcutaneously into a litter of newborn hamsters. No tumors appeared in 90 days at which time their sera had a HI titer of <20. At time of second transfer the cell suspension was inoculated into newborn hamsters as well as into adults. Tumors appeared at the same time in different aged animals and both were successfully transferred to adult animals. In none of the transplant passages were metastases seen, although adjacent satellite tumors appeared in several hamsters after the primary was well developed. Once the tumors had grown beyond 2 inches in diameter, necrosis with hemorrhage usually ensued and this was the cause of death.

Included in Table I are the HI titers on animals used in the transplantation series. With but 2 exceptions, Transfer 3 and 4, the animals developing tumors from the transplants were negative for HI antibodies even at times when tumors were quite large.

Histological examination of the original and transplant passage #8 tumors was made by Dr. Samuel S. Spicer who described both as similar and either fibroma or fibrosarcoma in character.

(b) *Lack of immunity to transplanted tumors.* Minced tumor suspension from third transplant passage was inoculated into animals which received multiple doses of original C474 tumor, blood or skin; hamsters with a first, second or third transfer tumor; 2 hamsters hyperimmunized with polyoma virus; a hamster with a virus-induced tumor and normal adult hamsters. The results in Table II show that tumors appeared at 10 days in all animals and there was no marked difference in their growth. Table III shows results of another experiment in which an adult hamster previously hyperimmunized with polyoma virus and 2 normal adults received a suspension of transplant #6 tumor with some evidence that tumor growth in the immune animal was slower than in controls. Again, in those animals developing tumors, HI antibodies did not appear within 21 days after challenge at which time their transplanted tumors were quite large, except in those that already had antibodies before challenge.

(c) *Attempts to transplant hamster tumor in other species.* Tumor of transfer #3 was inoculated subcutaneously into the following adult animals: 2 white rats (0.2 ml), 2 guinea pigs (0.2 ml), 5 Swiss mice (0.05 ml),

TABLE II. Results of Challenge with Transfer #3 Tumor for Possible Immunity in Hamsters.

History before challenge	Size of tumor (inches) 10 days	28 days	HI antibody titer 0 day	21 days
6 doses whole blood from C474	⅛ × ¼	1 × 1	ND	< 40
Idem	½ × ¾	2 × 2	"	40
8 doses skin emulsion from C474	¼ × ¾	2 × 4	"	"
8 doses tumor C474; large IP tumor	¼ × ½	Dead	40	< 40
1 dose polyoma virus 2 mo prior	½ × ¾	2½ × 1½	>2560	>2560
Idem	¼ × ½	2 × 2½	"	"
P2 transfer of tumor cell suspension 1 mo before; large tumor of 3 wk duration	½ × 1	2 × 3	< 40	40
3 doses polyoma virus during preceding month	¼ × ½	1½ × 1½	>2560	>2560
Idem	⅛ × ⅜	1 × 1½	"	"
P3 transfer of tumor cells 3 wk before; tumor of 4 days' duration	⅛ × ¼	2 × 2	160	80
Inoculated with virus as newborn 3 mo before; large subcut. tumor	½ × 1	2 × 3	40	40
Normal	½ × 1	Killed 20 days	< 40	< 40
"	½ × ¾	2 × 2	< 20	*< 20

* On 41st day HI 160.

and intracutaneously into 1 monkey (0.05 ml) as well as into 2 hamsters (0.2 ml). No evidence of tumor developed in rats, guinea pigs or monkey in 90 days' observation and no HI antibodies developed in the first 21 days, whereas (Table I) Hamsters 2030 and 2027 developed tumors in 10 days. In all 5 adult mice, millet seed nodules could be palpated at site of inoculation by 10 days but these never progressed except in one mouse where by 26 days a mass approximately 7 mm in diameter had developed. All mice were negative for HI antibodies at 3 weeks. At 26 days, the mass in the 1 mouse was removed and trypsinized and the resultant single cell suspension inoculated into 5 adult mice and 2 adult hamsters. In 60 days no tumors appeared in the mice but the hamsters developed progressively growing tumors starting in 12 days. At 21 days 1 of these tumors was removed, minced and inoculated into 5 adult mice which remained normal for 40 days. The original mouse tumor was also grown in tissue culture where the cells were definitely smaller than in tissue cultures of hamster tumors. This tissue culture was tested for virus by mouse inoculation test over the 42 days of its maintenance with negative results. It is of interest that of 2 hamsters developing tumors on transfer of the mouse tumor cells, one killed at 21 days had a negative HI test but the other at 40 days had a titer of 160.

(d) *Tissue culture of transplanted tumor.* Tumor from hamster 2030 representing transfer #4 was trypsinized and established in tissue culture as were also the tumors produced in newborn hamsters after inoculation with transfer #1 cells. When the cells in these tissue cultures had grown out they were trypsinized and divided into 2 flasks for subculture. One of each subculture level was maintained without further passage. The cultures were held for 48 days (4 subcultures) and 55 days (5 subcultures) from their inception and supernates at the time of biweekly medium changes were tested for the presence of virus

TABLE III. Effect of Polyoma Virus Immunity on Progression of Transplanted Tumor in Hamsters.

History before challenge*	Size of tumor (inches) 10 day	16 day	29 day	HI antibody titer 0 day	Day after challenge
Normal	½ × ½	1 × 1		< 20	16 – <20
"	½ × 1	1 × 2	3 × 4	"	29 – "
Hyperimmunized with virus	¼ × ¼	¼ × ½	1 × 1½	>2560	>2560

* Challenged with tumor cell suspension of transplant passage #6.

by inoculation into mouse embryo cultures or into mice with completely negative results. When they were respectively 40, 30 and 23 days old, passage 2, 3 and 4 subcultures of 2030 tumor were trypsinized, pooled, and a suspension of cells inoculated into an adult hamster in which a rapidly progressing tumor appeared at 14 days. This hamster's serum had an HI titer of <20 at 27 days after receiving the cell inoculum.

Discussion. Ability of this polyoma virus induced fibrosarcoma to be transplanted into adult hamsters through 8 transplants establishes one additional criterion concerning its malignancy. The infrequency of development of anti-polyoma virus antibodies in those animals supporting growth of large transplanted tumors and the fact that presence of high antibody levels against the virus did not prevent a take of transplanted cells, raise questions concerning relationships between virus and tumor. Others have found that it is difficult to isolate the virus from tumors developing after infection of newborn animals but this has been explained as due to presence of high levels of antibody in serum and tumor at time of its emulsification. However, this cannot be the explanation in animals carrying transplanted tumors since no antibody was demonstrable at the time the tumor was harvested. Furthermore, on carrying the transplanted tumor through 5 tissue culture transfers over 55 days, no evidence of virus could be demonstrated. Yet virus was present in the original tumor since it was isolated from the second and third tissue culture transfer of that tumor. The late development of antibody in a few hamsters in the transplantation series suggests that virus is present in the tumors but is released very slowly and in amounts insufficient to act as an effective antigenic stimulus to the animal's immunological system.

Summary. A fibrosarcoma produced by inoculation of polyoma virus into a suckling hamster has been transplanted through 8 transfers in adult hamsters with preservation of its original histological character. In only an occasional animal supporting a transplanted tumor have anti-polyoma virus antibodies appeared. The transplanted tumor was carried through 5 tissue culture transfers without demonstration of virus release and tissue culture cells were capable of producing tumors in an adult hamster. After 26 days sojourn in an adult mouse, the transplanted hamster tumor still produced tumors in the hamster but was not transplantable in series in the mouse. The presence of tumors or high level of virus antibodies did not prevent a positive take with the transplantable tumor.

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Received June 29, 1959. P.S.E.B.M., 1959, v102.

Adenylic Acid and Adenosine Deaminase Activities in Rat Livers During Azo-Dye Carcinogenesis.* (25157)

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Adenylic acid and adenosine deaminases are 2 purine-metabolizing enzymes. The former catalyzes conversion of 5′-adenylic acid to 5′-inosinic acid while the latter catalyzes conversion of adenosine to inosine(1). Recently, it was shown that adenylic acid de-

* The authors express their appreciation to Bettye Cox for technical assistance.

TABLE I. Effect of 3′-ME-DAB Feeding on Adenosine and Adenylic Acid Deaminase Activities in Precancerous Livers.*

	μM NH_3/mg N/15 min.			
	Adenosine deaminase		Adenylic acid deaminase	
Days	Control	Dye-fed	Control	Dye-fed
0	.40		.46	
30	.37	.93	.45	.64
60	.52	1.01	.44	1.31
90	.39	1.06	.37	.89

Adenosine deaminase: Adenosine, .025 M, phosphate buffer, .05 M, pH 7.0; tissue concentration, 30 mg (wet wt)/ml; 38°C.

Adenylic acid deaminase: Adenylic acid, .006 M, citric acid buffer, .05 M, pH 6.5; tissue concentration, 30 mg (wet wt)/ml; 38°C.

* Activities were determined on liver homogenates from 3 to 4 animals pooled.

aminase(2) and adenosine deaminase(3) activities in transplanted Novikoff hepatomas were considerably higher than normal liver. On the other hand, Reid and Lewin(4) found no consistent change in adenosine deaminase activity in azo dye-induced primary hepatomas. Since the transplanted Novikoff hepatoma was initially induced by an azo dye (5), it was our purpose to determine whether increases in adenosine and adenylic acid deaminase activities observed in transplanted hepatomas were the result of transplantation or occurred during azo-dye carcinogenesis. Levels of the 2 deaminases were determined in precancerous livers and in primary hepatomas induced by feeding 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB).

Materials and methods. Female Holtzman rats were fed for 90 days with 3′-Me-DAB synthesized by the method of Giese, *et al.*(6) in a semi-synthetic, riboflavin-deficient diet described by Medes, *et al.*(7). Control rats received identical diet without carcinogen. Throughout the dye-feeding period, control and experimental animals were sacrificed at 30-day intervals and 3 to 4 livers were pooled for each enzymatic determination. After 90 days carcinogen was withdrawn from the diet and dye-fed animals were sacrificed individually when primary hepatomas were detected by palpation. The assay method for deaminases has been described previously(2). Tissue homogenates were incubated with either adenosine or adenylic acid and ammonia production was determined at intervals to follow extent of the reaction. Controls, in which either substrates or homogenates were omitted from the reaction mixture, were determined in each experiment and only negligible ammonia production was observed. The unit of enzymatic activity for both deaminases is expressed as μM of ammonia produced/mg homogenate nitrogen/15 minutes incubation.

Results. The effect of 90 day azo-dye regimen upon adenylic acid and adenosine deaminase acvtivities of precancerous livers is shown in Table I. While control livers exhibited essentially no change in deaminase activities throughout 90 days, activities of both deaminases in livers taken from dye-fed animals were elevated about 100% after 30 days and maintained this level throughout 90 days. Since pathological examinations† showed that precancerous livers were essentially free of neoplastic tissues throughout 90 days, it seemed reasonable to conclude that increases in deaminase activities had occurred prior to formation of neoplastic tissues.

When deaminase activities in primary hepatomas and liver tissues adjacent to hepatomas were determined and compared with control livers (Table II), activities in primary

TABLE II. Adenosine and Adenylic Acid Deaminase Activities in Primary Hepatoma and in Adjacent Liver.*

	μM NH_3/mg N/15 min.	
	Adenosine deaminase	Adenylic acid deaminase
Control	.34	.45
Primary	.81 ± .02†(2)‡	1.33 ± .45 (5)
Adjacent liver	.96 ± .36 (3)	.90 ± .07 (2)

Experimental conditions: See Table I.

* Control values from livers of 3 animals pooled; activities of primary hepatomas and adjacent livers were determined on individual animals.

† Stand. dev.

‡ No. of observations.

hepatomas and adjacent livers were considerably higher than control livers and were essentially the same as those in precancerous livers. From these data it appears that no additional change in deaminase activities occurred following formation of primary hepa-

† The authors express appreciation to Dr. E. S. Irvine for pathological examinations.

tomas. While adenosine deaminase data in our study did not correlate with observations of Reid and Lewin(4), DeLamirande and Allard‡ reported data which, in agreement with present findings, showed increased adenosine deaminase activity in precancerous livers from 3′-Me-DAB feeding.

Comparison of enzyme activities in liver and hepatomas led Potter(8) to formulate an enzyme deletion hypothesis in which it was suggested that loss of catabolic enzymes in tumor tissues functioned to preserve metabolites for synthetic pathways associated with cell multiplication. Activities of several purine-catabolizing enzymes, such as 5′-nucleotidases, 5′-nucleoside phosphorylases, guanase and adenase, were reported(3) considerably lower in Novikoff hepatomas compared with normal liver; while xanthine oxidase and uricase appeared to be absent. Decreases of these purine-catabolizing enzymes could be interpreted as functioning to preserve purines for nucleic acid synthesis. Since adenylic acid deaminase and adenosine deaminase may function in conversion of adenine compounds to guanine compounds for incorporation into nucleic acids, their increased enzymatic potential during azo-dye carcinogenesis may be correlated with the metabolite preservation concept of Potter(8). Moreover, it is interesting to note that studies with other fast growing tissues such as chick embryos(9) and regenerating rat livers(10) also showed increased adenosine deaminase activity.

Summary. Adenosine and adenylic acid deaminase activities were determined in precancerous livers and in primary hepatomas induced by feeding 3′-methyl-4-dimethylaminoazobenzene. The deaminase activities in precancerous livers were increased about 100% after 30 days of dye feeding compared with control livers. Activities in primary hepatomas and in the liver adjacent to primary hepatomas were essentially the same as that of precancerous livers, indicating that no additional changes occurred upon formation of neoplastic tissues.

‡ This report was presented at Am. Assn. for Cancer Research, Atlantic City, N. J., 1959.

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Received June 29, 1959. P.S.E.B.M., 1959, v102.

Determination of Apparent Hippuric Acid and Free Glycine in Urine by Microbiological Assay.* (25158)

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Values for "bound" or "combined" amino acids in urine have commonly been reported as the difference between amino acid levels found by microbiological assay of this material before and after subjecting it to acid hydrolysis (1-4). It has generally been recognized that such values may lack quantitative significance,† but that they may not be meaningful

* Paper 128. This work was aided by grants from U.S.P.H.S., Am. Cancer Soc., and Univ. of California. Authors are indebted to Evelyn Brown for technical assistance.

† Qualifying quotation marks are almost always used with the terms "free" and "bound" in this sense.

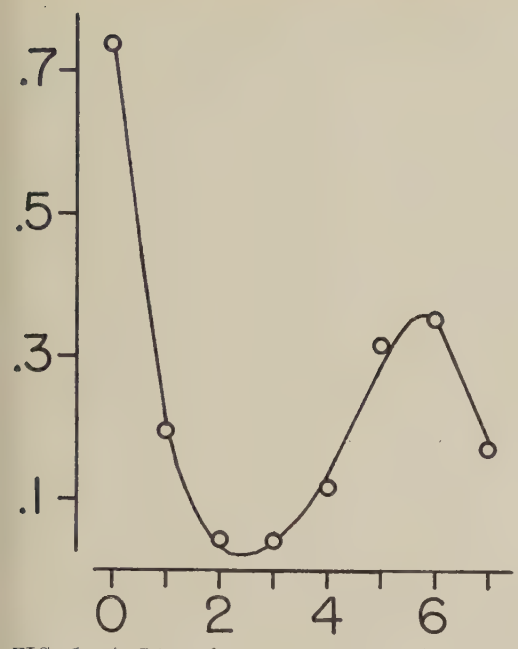

FIG. 1. A 7-transfer countercurrent distribution of glycine and hippuric acid with *n*-butanol and 5% hydrochloric acid. Cell numbers are indicated on horizontal scale. Fractions of total glycine (left hand peak) and hippuric acid (right hand peak)/cell are indicated on vertical scale. Plotted values were calculated from titrations of residual glycine hydrochloride and hippuric acid with sodium hydroxide after evaporating cell contents to dryness. Curve drawn through the points is the theoretical one (derived mathematically) for 2 components with partition ratios of 0.04 (left hand peak) and 3.7 (right hand peak), respectively.

has not been so widely appreciated. Microbiological assay of glycine in urine before and after hydrolysis, for example, yields values (5-7) which give no indication whatever of the relative amounts of free and bound glycine. The reason is that hippuric acid, a major bound form of urinary glycine, effects a microbiological response, qualitatively and quantitatively similar to that of free glycine (8). The present report concerns separation of hippuric acid from free glycine in urine before subjecting it to microbiological analysis. This modification of the usual glycine assay procedure was developed as a means of furthering current investigations of glycine and benzoic acid metabolism in the authors' laboratory.

Methods. Countercurrent extraction procedure and solvent system were those described by Craig *et al.*(9). The partition ratios of glycine (0.04) and hippuric acid (3.7) in this system (*n*-butanol, 5% hydrochloric acid) permitted quantitative separation of these metabolites with a 7-transfer distribution (Fig. 1). Eight cells of conventional countercurrent extraction train(9) were employed for this trial separation (Fig. 1), although 4 of 8 cells were eliminated in subsequent separations by employing "single withdrawal" procedure(9). Centrifuge tubes (narrow neck with rubber stoppers) were used as extraction cells to distribute the urine samples‡ because this permitted rapid separation of emulsified solvents by centrifugation. In these cases the lower phase was taken as the mobile phase because it proved most convenient of the 2 phases to transfer by pipet from one tube to the next. Fresh lower phase was added to cell 0 (first tube in the series) after the first through third transfer. Lower phase was withdrawn from cell 3 (last tube in the series) after fourth through seventh transfer.§

‡ Pooled urine with and without measured additions of glycine and hippuric acid (for recovery tests) was acidified to contain 5% hydrochloric acid and saturated with *n*-butanol before subjecting it to the described countercurrent extraction.

§ Tubes were shaken well and centrifuged (if required to separate the phases) before each transfer. Initially, 15 ml of fresh upper phase solvent (*n*-butanol saturated with 5% hydrochloric acid) was placed in each of the 4 cells, and 15 ml of urine sample was placed in cell 0. The first transfer was accomplished by pipetting lower phase from cell 0 into cell 1 and adding 15 ml of fresh lower phase solvent (5% hydrochloric acid saturated with *n*-butanol) to cell 0. The second transfer was accomplished by pipetting lower phase from cell 1 into cell 2 and from cell 0 into cell 1 and adding 15 ml of fresh lower phase solvent to cell 0. The third transfer was accomplished by pipetting lower phase from cell 2 into cell 3, from cell 1 into cell 2, and from cell 0 into cell 1 and adding 15 ml of fresh lower phase solvent to cell 0. The fourth transfer was accomplished by pipetting lower phase from cell 3 into a collecting flask (to contain the "free-glycine fraction"), from cell 2 into cell 3, from cell 1 into cell 2, and from cell 0 into cell 1. This procedure was repeated until 7 transfers were completed and a total of 4 lower phase portions from cell 3 were collected in the collecting flask. Fresh lower phase was not added to cell 0 after fourth

TABLE I. Glycine in Urine Fractions.*

Addition to sample, μM/ml	Fraction assayed†	Glycine found,‡ μM/ml	Glycine recovered§ μM/ml	%
None	F.G.	3.07 (2.93-3.14)		
	H.A.	4.62 (4.60-4.65)		
Glycine 4.28	F.G.	7.16 (7.00-7.28)	4.09	95.6
	H.A.	4.68 (4.57-4.77)	.06	1.4
Hippuric acid 3.55	F.G.	3.06 (2.83-3.40)	.0	.0
	H.A.	8.04 (7.86-8.28)	3.42	96.3

* Equal volumes of urine from 5 apparently normal male volunteers were pooled. Aliquots of combined material were supplemented as indicated (column 1 of Table) and subjected to fractionation and assay procedures given in text.

† F.G. = "free glycine fraction;" H.A. = "hippuric acid fraction."

‡ Each value is avg (range in parentheses) of 4 independent assay results.

§ Includes glycine added as hippuric acid.

The combined lower phase solutions withdrawn from cell 3 were taken as the "free glycine fraction," and the combined upper phase solutions remaining in the 4 cells were taken as the "hippuric acid fraction." Both fractions were freed of butanol by distillation, and the aqueous residues were subjected to acid hydrolysis and microbiological assay for glycine essentially as described previously(5). Results are compiled in Table I.

Discussion. The experimental data (Fig. 1, Table I) suggest that the abbreviated countercurrent extraction procedure described here is capable of effecting a quantitative separation of free glycine from hippuric acid in urine as well as in artificial mixtures of these metabolites. The apparent ratio of hippuric acid to free glycine in normal urine is approximately 1.5 : 1 according to these data. It is evident that the apparent ratio could deviate considerably from the true ratio if either fraction of urine sample should contain some bound form of glycine other than hippuric acid. It seems likely, however, that despite this potential source of error, determination of apparent free glycine and apparent hippuric acid in this manner can provide useful information for metabolic, clinical and nutritional studies, particularly those directly concerning glycine and benzoic acid.

Classical data for hippuric acid in normal urine yield the average value of 3.3 μM/ml,‖ with "normal" concentrations ranging to several times the average under the influence of dietary factors(10). The present value of 4.6 μM/ml (Table I) is therefore in reasonable accord with the classical data. It is of interest that total glycine (including hippuric acid glycine) ranged from 3.1 to 11 μM/ml (median 6.8 μM/ml) in fourteen 24-hour urine samples from 6 normal subjects on free-choice diets and from 11 to 17 μM/ml (median 15 μM/ml) in eight 24-hour urine samples from one of the same individuals maintained solely on army "K-ration" according to earlier data from this laboratory(5).

Seemingly reliable literature data for free glycine in urine appears limited to those obtained by chromatographic procedures, and the values reported on this basis for normal urine(11-13) vary from 0.3 to 1.8 μM/ml (range of 13 values, median 1.3 μM/ml). The discrepancy between these values and the present value of 3.1 μM/ml (Table I) suggests that "apparent free glycine" determined by the present method may include considerable amounts of combined glycine (other than hippuric acid). It is of interest that one author(11) has reported the presence of 2 glycine-containing peptides at concentrations totalling approximately 0.3 μM/ml in normal urine. It is possible, on the other hand, that "normal" values for free glycine in urine may actually range to levels considerably higher than those reported.

The solvent system and simple stage by stage extraction described here have proved satisfactory for separation of hippuric acid from free glycine in urine. Other solvent systems and extraction procedures might nevertheless be advantageous. Preliminary tests

and subsequent transfers since such additions would not be carried beyond cell 3 by these transfers and would therefore be superfluous.

‖ Calculated from 0.7 g hippuric acid and 1200 ml water, reported as average 24 hour normal urine values by Hawk *et al.*,(10) (and in most other physiological chemistry text books).

of ether *vs.* dilute hydrochloric acid and ethyl acetate *vs.* dilute hydrochloric acid, for example, yielded partition ratios of 0.3 and 2, respectively, for hippuric acid, but less than 2 x 10^{-7} and 4 x 10^{-4}, respectively, for free glycine. These data suggest that quantitative separation of hippuric acid from glycine could be effected by either carefully controlled continuous liquid-liquid extraction with either of these solvent systems¶ or appropriate stage by stage extraction from a single cell with ethyl acetate versus dilute hydrochloric acid system.

Summary. Apparent free glycine and apparent hippuric acid in urine have been determined by microbiological analysis after fractionating it by a 4-cell, 7-transfer countercurrent distribution between *n*-butanol and 5% hydrochloric acid. The effectiveness of this procedure was demonstrated by separating hippuric acid from free glycine in known mixtures and by recovering known amounts of these metabolites added to urine before subjecting it to the fractionation procedure. The apparent concentrations of free glycine and hippuric acid in normal pooled urine were 3.1 and 4.6 μM/ml, respectively.

¶ Earlier investigators employed continuous liquid-liquid extraction with ethyl acetate(14) and ether (15,16) in connection with chemical determinations of urinary hippuric acid.

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Received June 29, 1959. P.S.E.B.M., 1959, v102

A Spectrophotometric Technic for Measuring Erythrocyte Chimerism in Cattle.* (25159)

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The vascular anastomosis known to occur frequently between pairs of cattle dizygotic twins(1) permits an interchange of blood-forming tissues resulting in chimerism of the erythrocytes(2). The 2 kinds of erythrocytes within each member of such twin pairs can be demonstrated by a technic known as differential hemolysis(3,4). Generally, cells of an individual sensitized by an appropriate antibody (reagent) are completely hemolyzed by addition of complement (normal rabbit serum) (5). However, cells of chimeras often are only partially hemolyzed by this treatment. Such partial reactions indicate presence of 2 distinct cell populations, those with and without the antigenic specificity against which the reagent reacts. Complete antigenic constitutions of the 2 cell types can usually be determined by testing residual cells (unhemolysed)

* Paper No. 741 Dept. of Genetics. Published with approval of Director of Agri. Exp. Station, Univ. of Wisconsin. This project supported in part by Research Committee of Graduate School from funds supplied by Wisconsin Alumni Research Fn.

with a battery of blood typing reagents. The proportion of the 2 cell types can be determined by measuring the fraction of cells not lysed: Owen(3) obtained reasonably precise results using a microscopic cell counting technic, but this method was lengthy and tedious, and required as much as 4-6 ml of reagent. More recently Rendel(4) obtained comparable results using hematocrit tubes. This method was much less time-consuming, but did not conserve on reagent. Precision was limited, especially when the fraction of residual cells was small. This report describes a new technic for determining the fraction of residual cells by measuring transmittance of hemoglobin solutions derived from cell samples before and after treatment with those reagents yielding partial hemolysis. This method gives reproducible results, conserves on reagent, and requires little more time than the hematocrit technic.

Materials and methods. Blood was drawn from the animals into tubes containing 2% sodium citrate. Erythrocytes were washed 4 times in saline (0.9%) and final suspension adjusted so that ½ ml of cell suspensions lysed by diluting to about 4 ml with distilled water gave about 25% transmittance in 13 x 100 mm tubes. From knowledge of blood types, the antigenic factors for which the animals were probably mixed were determined and appropriate reagents selected for use. The reagents were used at a concentration that would give complete hemolysis with known positive cells within 4 hours' incubation at room temperature (24 ± 4°C) in a standard hemolytic test(5). Ingredients for differential hemolysis tests were added to 13 x 100 mm tubes (matched for the spectrophotometer and marked at 3 ml) according to the following Table:

	Exp. tubes	Complement control tubes	Saline control tubes
No. of tubes	1/reagent	3 (total)	3 (total)
Erythrocyte suspension, ml*	.50	.50	.50
Saline, ml		1.0	1.5
Reagent, ml	1.0		
Complement (undiluted), ml	.5	.5	

* Volumetric pipette.

The tubes were twirled at frequent intervals during incubation. Cells remaining after treatment with reagent and those in control tubes were washed 4 times in saline. The pellet of cells obtained was then lysed by diluting to the 3 ml mark with distilled water. Tubes were capped to prevent evaporation and centrifuged 20 minutes at 3000 rpm to sediment the stroma. Tubes were wiped clean and transmittance of each read twice with a Bausch and Lomb *Spectronic 20* set at 550 mμ. Percentage cells remaining was calculated from the formula $P = 100 \frac{\log T_e}{\log T_c}$ where T_e is average transmittance (expressed as decimal fraction) of those tubes yielding approximately the same result, and T_c is average transmittance of complement control tubes. Since complement is not entirely non-lytic, complement control tubes represent more accurately than saline control tubes the base for calculating percent cells remaining. If complement were non-lytic the formula $P = 100 \frac{\log T_e}{\log T_s}$ would be correct. T_s is average transmittance of saline control tubes. Under the assumption that complement acts uniformly upon only the cells remaining after lysis by reagent, it can be shown that this formula transforms into the formula stated before. If the 2 cell types do not occur in approximately equal proportions, T_e values of the experimental tubes fall into 2 distinct groups defined by antigens unique to each of the cell types in the chimera. However, when the 2 cell types occur in approximately equal proportions, it is not possible to determine immediately which T_e values should be grouped for averaging. The residual cells must then be blood typed to delineate the 2 groups. Two additional groups which yield no information (but are useful indicators of correct procedure) are defined by antigens common to both types ($P = O$), and lacking on both types ($P = 100$). The computed value of P is subject to error due to errors in measurements of transmittance. An estimate of this error (E) was obtained by differentiating the equation for P, and substituting for the differential terms the standard deviations of $\log T_e$ and

TABLE I. Percentage Admixture in One "Artificial Twin" and 4 Naturally Occurring Twin Sets Showing Erythrocyte Chimerism.

Twin set	Cell type	% of each cell type in twin a	b	Antigenic constitution by loci (abbreviated) AH	B	C	FV	L	SU	Z	Reagents used specific for cell type I	II
A1* (1st test)	I	28.0 ± 3.0†		—/—	$B_1O_1/$—	$C_1WX_3/X_2L'E$	V/V	—/—	—/—	Z/—		
	II	63.1 ± 3.8		—/—	$GY_2E_1'/O_3J'K'$	C_1X_3E	F/F	,,	,,	—/—	$B_2L'Z$	Y_2E_3'
A1* (2nd test)	I	30.8 ± 1.2				as above					,,	(J'K')
	II	66.9 ± 1.6										
N1	I	12.7 ± .6	20.3 ± .6	A /—	$B_1O_1I'/Y_1D'E_3'$	C_2RX_1E/WX_2	F/V	,,	S /—	Z/Z	$B\,I'\,V$	$E_1'\,E_2'\,E_2'$
	II	84.8 ± 1.7	79.0 ± 2.2	,,	$QD'E_1'/Y_1D'E_3'$	,,	F/F	,,	,,	Z/—		
N2	I	5.0 ± .3	7.1 ± .3	,,	B_1O_1D'/GY_2E_1'	$C_1X_1/$—	F/V	,,	,,	,,	$A\,B\,G\,Y_2$	J'K'
	II	95.8 ± 1.8	92.9 ± 2.0	—/—	$D'E_3'/O_3J'K'$	,,	F/F	,,	—/—	,,	$E_2'\,V\,S$	(,,)
N3	I	40.2 ± 1.2	45.5 ± 1.0	A /—	$B_1GKO_3Y_2A'/O_1A'$	C_1W_2E	F/F	,,	SU_2	—/—	$B\,G$	$D'\,I'\,Q$
	II	58.6 ± 2.7	56.9 ± .5	,,	$Y_1D'T'/Q$	X_2E	F/V	L /—	,,	,,	$C_1\,W_2$	$X_2\,V\,L$
N4 (1st test)	I	28.8 ± 3.6	42.6 ± 1.5	,,	$L_2/$—	C_1E	F/F	,,	—/—	Z/—		
	II	77.2 ± 1.7	61.9 ± 1.2	,,	,,	C_1X_3E	F/V	—/—	,,	—/—	$L\,Z$	$X_2\,V$
N4 (2nd test)	I	26.4 ± 1.2	39.1 ± 3.2			as above						
	II	75.5 ± 2.6	61.1 ± 2.9									

* Actually mixed 33% cell type I and 67% cell type II.

† E value, see text.

$\log T_c$. Denoting these quantities by S_e and S_c respectively the value of this "propagated standard deviation in P" due to errors in measurements is given by:

$$E = 100\left[\frac{1}{\log T_c}S_e + \frac{\log T_e}{(\log T_c)^2}S_c\right]$$

Results. The results of differential hemolysis tests are reported here for 2 tests on an "artificial twin" (made by mixing cells from 2 different cows), and for 4 naturally admixed twin sets. The artificial twin was made by combining 1 part of cell type I with 2 parts of cell type II. Results of all tests are summarized in Table I and Fig. 1. Proportion of the 2 cell types determined by tests on the artificial twin (Twin A1) closely approximated the proportions in which the 2 cell types were originally mixed, suggesting that the technic gave reasonably accurate results. Evidence for reproducibility was provided by results of duplicate tests on both artificial twin (A1) and natural twin (N4). The results of first and second tests were similar for both sets of twins.

Among the majority of twin pairs, each member appears to have approximately the same proportion of the 2 cell types as its cotwin. Exceptions to this generality have been noted by Rendel(4), and further examples are afforded by twin sets N1 and N4.

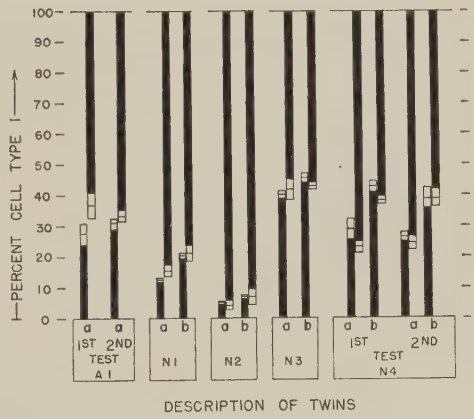

FIG. 1. Percentage admixture of 2 cell types in cattle chimeras. A1 is an "artificial twin" mixed 33% cell type I, 67% cell type II. N1, N2, N3 and N4 are natural twin sets showing chimerism. Open bars represent computed percentages plus and minus E, "propagated stand. dev. in P."

Discussion. The method of measuring the percentage admixture in cattle chimeras presented here is less tedious than cell count methods, has greater precision and is no more tedious than hematocrit methods, and requires much less reagent than either of these methods. All 3 methods depend upon the measurement of the percent cells remaining after hemolysis of one or the other cell types by appropriately chosen reagents, and each is subject to certain errors in this measurement. The spectrophotometric method requires that bovine oxyhemoglobin follow the Beer-Lambert Law of absorption, and that solutions of oxyhemoglobin in distilled water be stable in time with respect to absorption. Both these conditions have been carefully checked and found satisfactory.

By extensions of this method it should be possible to perform the differential hemolytic tests with less than 1.0 ml reagent (thus conserving further), and to measure the percent cells hemolyzed, as well as percent cells remaining, thus gaining twice as much information per test with little more work. Owen(6) used a spectrophotometer to measure transmittance of hemoglobin solutions after differential hemolyses of the red blood cells of irradiated mice injected with homologous erythropoietic tissue. It should be possible to apply our technic to studies of human blood cell chimeras(7) using a hemolytic rather than a hemagglutination system.

Summary. An easily performed and precise method for measuring degree of erythrocyte chimerism in cattle is presented. A spectrophotometer is used to measure transmittance of solutions containing hemoglobin of those cells remaining after differential hemolysis by appropriately chosen reagents.

The authors wish to thank Janis Beckstrom for performing several of the differential hemolytic tests.

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Received July 1, 1959. P.S.E.B.M., 1959, v102.

Effect of Secretin Given into Portal or Peripheral Vein.* (25160)

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Intravenously injected secretin is considered generally to equal the physiological effect of the hormone as liberated during digestion. However, there is reason to assume a difference in effect on the pancreas between these 2 ways of entrance of the hormone into the circulation, because a number of hormones seem to be inactivated in the liver. The naturally secreted secretin most probably enters the portal circulation and passes through the liver first, while in intravenous injection it passes the liver only after it has been diluted in the general circulation(1). This concept is supported by results obtained with vivdialysis by Necheles and Lim(2). Previous workers, using rather crude preparations of secretin, found a weaker response of pancreatic secretion following its injection into the portal vein than into a systemic vein(3). However, Mellanby(1) using a more purified secretin, found no such difference. Our attention was focused on this question when we were able to obtain a highly purified secretin labelled with I^{131}†; following its intravenous

* Supported by grant from Merton Davis Club.

† I^{131} kindly supplied by S. Bocchieri, Abbott Radio Pharmaceuticals, Oak Ridge, Tenn, and Dr. R. K. Richards, Abbott Labs., North Chicago, Ill. Secretin was kindly supplied by Dr. R. M. Rice, Lilly Lab. for Clin. Res., Product E. G.-110-33A.

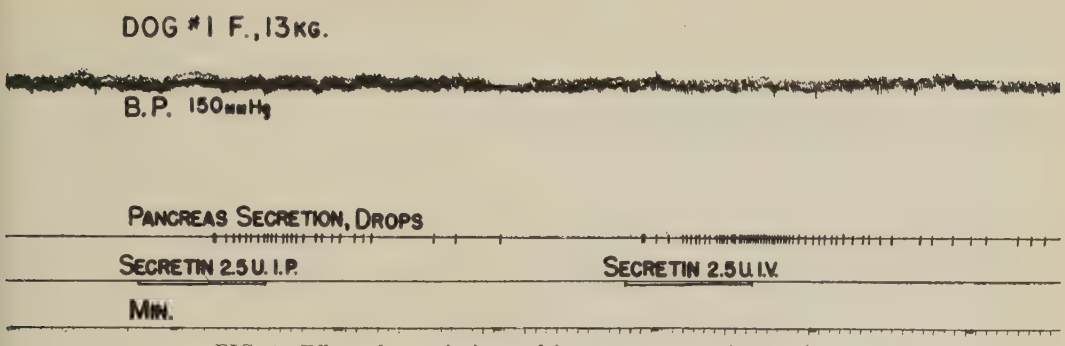

FIG. 1. Effect of secretin i.v and i.p. upon pancreatic secretion.

injection, we found only low concentrations of I^{131} in the pancreas and high concentrations in the liver. We assumed this to indicate a primary concentration of secretin in the liver and possibly a secondary release into the circulation and to the pancreas. Furthermore, we believe that the highly purified secretin that is available now may yield more valid results than preparations made before 1926.

Methods. Adult male and female mongrel dogs were anesthetized with intravenous Nembutal; cystic duct, accessory pancreatic duct, and pylorus were ligated, a plastic tube was secured in the main pancreatic duct and connected to an electronic drop recorder.‡ Carotid blood pressure was recorded with a mercury manometer. A fine polyethylene tube was introduced through mesenteric vein into the portal vein, and a peripheral vein, usually femoral, was cannulated. The animals were starved for approximately 16 hours, except 4 (No. 8-11 on Fig. 1) that were starved for longer periods of time. Doses of 2 to 10 units of secretin were given into each vein alternately (i.v. or i.p.). A total of 13 experiments were conducted on 11 dogs. Only such experiments are reported in which the blood pressure of the animal was stable above 110 mm of mercury (110-160 mm Hg). Secretin i.v. or i.p. did not affect blood pressure or respiration.

Results. In Table I in 11 out of 13 tests i.p. injection of secretin had distinctly less effect on pancreatic secretion than i.v. injection. The peak of secretion was reached in the first 10 minutes after secretin injection in most experiments, with little difference between the type of response to i.v. and i.p. administration, such as latent period or duration of secretion (Fig. 1). The peaks of secretory response to i.v. and i.p. injection of secretin were compared. If the response to i.v. secretin is 100%, the response to i.p. administration varied between 28 and 109% (above 100% in one animal only), with an average of 69.5% (stand. dev. ± 21.1); the difference is statistically significant. In one experiment in which secretin was injected into a major pancreatic artery, the response of the pancreas was very large. Two units of secretin i.v. did not stimulate the level of pancreatic secretion above control values (5 and 4 drops respectively in 30 minutes), while intra-arterial injection of 2 units of secretin produced a secretion of 62 and 46 drops respectively in 30 minutes.

Discussion. We know that a number of substances are inactivated in the liver. Among these most probably are substances absorbed

TABLE I. Pancreatic Secretion to I.V. and I.P. Secretin in 13 Experiments.

Secretin dose in mg	No. of drops—10 min. of max secretion I.V.	I.P.	I.P./I.V.
5	18	11	.61
5	41	27	.66
2.5	29	15	.52
5	92	88	.96
5	99	86	.87
5	10	6	.60
10	11	12	1.09
9	35	19	.54
9	81	23	.28
3	20	12	.60
3	30	21	.70
3	19	15	.79
3	17	14	.82
		Mean: .695 (±.211)	

‡ Elmac Engineering Co., Chicago, Ill.

from or liberated in the gastrointestinal tract (4). An enzyme(secretinase,5,6) which destroys secretin has been described and it is possible that it is present in the liver. Another possibility is that secretin is modified in the liver or stored there and released later. We conclude that the same amount of secretin given into the portal circulation does not have the same effectiveness in inducing pancreatic secretion as when administered into a peripheral vein. Secretin is considered(7) to play a role in neutralization of gastric HCl in the upper small intestine, but the results of our present work indicate that this mechanism may be less effective than hitherto believed.

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Received July 1, 1959. P.S.E.B.M., 1959, v102.

Adrenal Cortex and Frog Skin Potentials.* (25161)

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Previous reports demonstrate that in the wake of adrenocortical insufficiency there are alterations in size, shape and conduction velocity of spike potentials in frogs(1), in electrocardiograms(2), and electroencephalograms of humans(3), in electroencephalograms and spinal-cord conduction time and in nerve excitability of rats(4). The present study considers bioelectric potentials of the ventral skins taken from frogs previously subjected to varying concentrations of adrenal cortical substance as effected by adrenalectomy and by administration of whole adrenal cortical extract (ACE) to otherwise normal frogs.

Material and methods. Male 20-40 g frogs, *Rana pipiens,* were divided into 4 groups: *A*) normal, *B*) adrenalectomized, *C*) renal-damaged and *D*) ACE-injected frogs. Operations for adrenalectomy and for renal damage were performed, and the animals used experimentally as previously reported(5). Group *D* was given 0.01 ml ACE (Upjohn) 3 times/week for 4 to 5 weeks. Unpigmented belly skin was removed, rinsed and immersed in 50 ml Ringer at 22 to 28°C. All Ringer solutions were oxygenated and buffered at pH 7.4 with $NaHCO_3$. Thirty-forty minutes after removal, a given skin was placed in a holder similar to that used by Steinbach(6). All potentials were determined to nearest 0.5 millivolt (mV) with a potentiometer and an H-S galvanometer as null apparatus. The electrode half-cell system was calomel-saturated-KCl/saturated-KCl/Ringer-agar-agar/Ringer //skin//, and so on. Two minutes prior to each 10-minute reading, both sides of skin were flushed from a common reservoir with volume of oxygenated Ringer equal to 2 to 3 times that of capacity of reservoir. Skin potentials were followed for 1½ hours; in many instances, longer. The potentials tended to stabilize within *ca.* 60 minutes (Fig. 1).

Results. Results are summarized in Fig. 1, where mean potential values in mV are plotted as functions of time in minutes. Letters and numbers appended to each curve designate the 4 frog groups and number of animals used/group. Mean potential values, with their respective standard errors, for various groups at end of 1 hour are: *A*) -31.2 ± 2.2 mV, *B*)

* Supported in part by grant from Comly-Coleman Fund for Medical Research, Ohio State Univ. College of Med.

† Now at College of Medicine, Univ. of Vermont, Burlington.

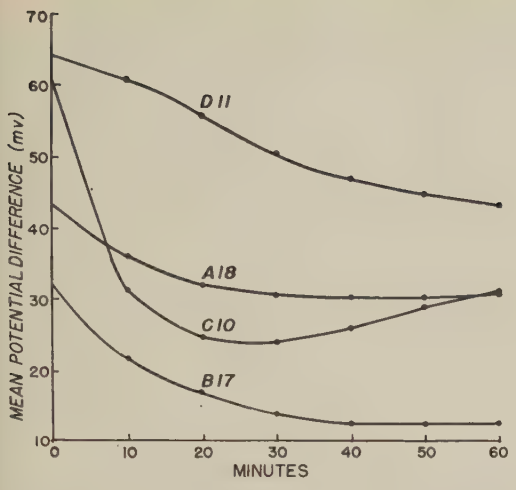

FIG. 1. Mean potential differences in millivolts across isolated ventral skins of common grass frog under experimental conditions indicated by appended letter over each curve: A) control, B) adrenalectomy, C) sham operated, D) injected with ACE, versus time in minutes. Numbers designate number of animals sampled.

-12.5 ± 1.7 mV, *C*) -34.4 ± 4.1 mV and *D*) -43.7 ± 4.6 mV. The following statistical comparisons are based on per cent difference between indicated means with their probability (p) values; $C - A = -0.3\%$ ($p > 0.5$), $B - C = -60.2\%$ ($p < 0.001$) and $D - A = +39.6\%$ ($p < 0.02$).

Discussion. "Normal" skin potentials range from –30 to –50 mV (outside surface is considered electronegative). These results are in agreement with those previously reported (6,7).

In evaluating mean skin potential for adrenalectomized frogs, that for renal-damaged frogs must be considered the control. A comparison of mean skin potentials for "normal" and renal-damaged frogs (*C-A*) shows essentially similar values. The 60% decrease in mean skin potential for adrenalectomized frogs is interpreted as due to adrenocortical insufficiency.

The decrease in skin potentials of adrenalectomized frogs suggests some fundamental change(s) underlying those physicochemical properties normally responsible for its production, *viz.*, metabolism and diffusion(6,7). In support of the metabolic theory are the observations that anoxia and metabolic inhibitors suppress frog skin potentials. Similarly, a decrease in adrenocortical secretions decreases the metabolism of mammals(8), frogs(9) and of certain mammalian tissues(8). It should be noted that anoxia resulting from circulatory failure concomitant with adrenocortical insufficiency(10) may be a contributing factor.

Were diffusive forces wholly or partially involved, there is evidence to suggest that adrenocortical insufficiency increases the permeability of tissue for water(11). This may involve an increase in plasma membrane permeability and/or an alteration in structure of intracellular cement. In support of the former, there is no direct evidence; in support of the latter, some studies indicate that the adrenal cortex has a profound inhibitory effect on substrates (mucopolysaccharides) acted upon by hyaluronidase(12). These substrates may well be the important constituents of the intracellular cement(13).

At the end of one hour the mean skin potential for ACE-injected frogs showed a significant increase when compared to those for normal frogs. Skin potentials for placebo-injected frogs gave results similar to controls; therefore, the action appears to be attributable to injected ACE. One would anticipate that the same mechanism which operated to decrease potentials during adrenocortical insufficiency also served to increase them as a result of an increase in ACE level, but this may not necessarily be the case.

Summary. Potential differences measured across ventral skin of the frog were studied under the influence of varying levels of adrenal cortical hormones. Increased levels of adrenal cortical extract added by injection produced a significant rise in potential difference across the frog skin, while decreased levels of adrenal cortical hormones produced by adrenalectomy significantly reduced the potential difference across the frog skin. Sham operated animals were not significantly different from unoperated controls.

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Received July 6, 1959. P.S.E.B.M., 1959, v102.

Spontaneous Hemorrhage Induced by Administration of Aminoacetonitrile and Papain or Anticoagulants to Rats. (25162)

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The principal components of the matrix of epiphysial cartilage are collagen and chondroitin sulfuric acid-protein complex. Certain agents will damage, possibly selectively, each of these constituents. The lathyrogenic agent, aminoacetonitrile (AAN), appears to interfere with polymerization of tropocollagen molecules to collagen fibrils(1); hence, although total collagen content of cartilage from AAN-treated animals is not reduced, the physical properties of such tissue are greatly altered. Intravenous administration of crude papain leads to liberation of chondroitin sulfate from certain tissues, including cartilage (2), possibly as a result of enzymatic destruction of the protein to which the polysaccharide is bound(3). It was thought that the simultaneous effects of the 2 agents, AAN and papain, on cartilage, particularly reparative sequences, might be of interest. During the course of these studies, to be reported elsewhere, widespread, spontaneous subperiosteal hemorrhage has been observed. An exploration of the pathogenesis of this disease state is the subject of this report.

Materials and methods. Albino rats, weighing 45 to 50 g, were used in all experiments. Aminoacetonitrile hydrosulfate, generously supplied by Dr. I. V. Ponseti, was administered by stomach tube in either 1 dose of 20 mg or 2 doses of 10 mg/day. Crude papain was made as a 5% solution in physiological saline and filtered. The usual dose was .5 cc injected into femoral vein. Heparin sodium was administered intravenously, .5 mg being the usual amount. Sodium polyglucose sulfate, generously supplied by Dr. Peter Mora, was administered intravenously, 5 mg being the usual dose. Dicoumarol was added to ground laboratory chow to make a concentration of .1%. Care was taken to cauterize the site of the intravenous injection of papain or anticoagulants lest the animal die of hemorrhage. At autopsy blocks of tissue were fixed in neutral formalin and processed in standard fashion.

Observations. When AAN is administered to normal rats, profound effects are found in rapidly growing epiphysial cartilages in 24 to 48 hours(1). At this time the periosteum may easily be detached from the tibia or calvarium. Gross hemorrhage in these areas is not common at this time, nor is bleeding conspicuous about the ribs, though microscopic subperiosteal hemorrhages are often seen after 2 to 3 days' administration of AAN.

If crude papain is administered intravenously to animals which have received AAN for 1 or 2 days, the rats become listless after 1 to 2 hours. The coat becomes ruffled; in a

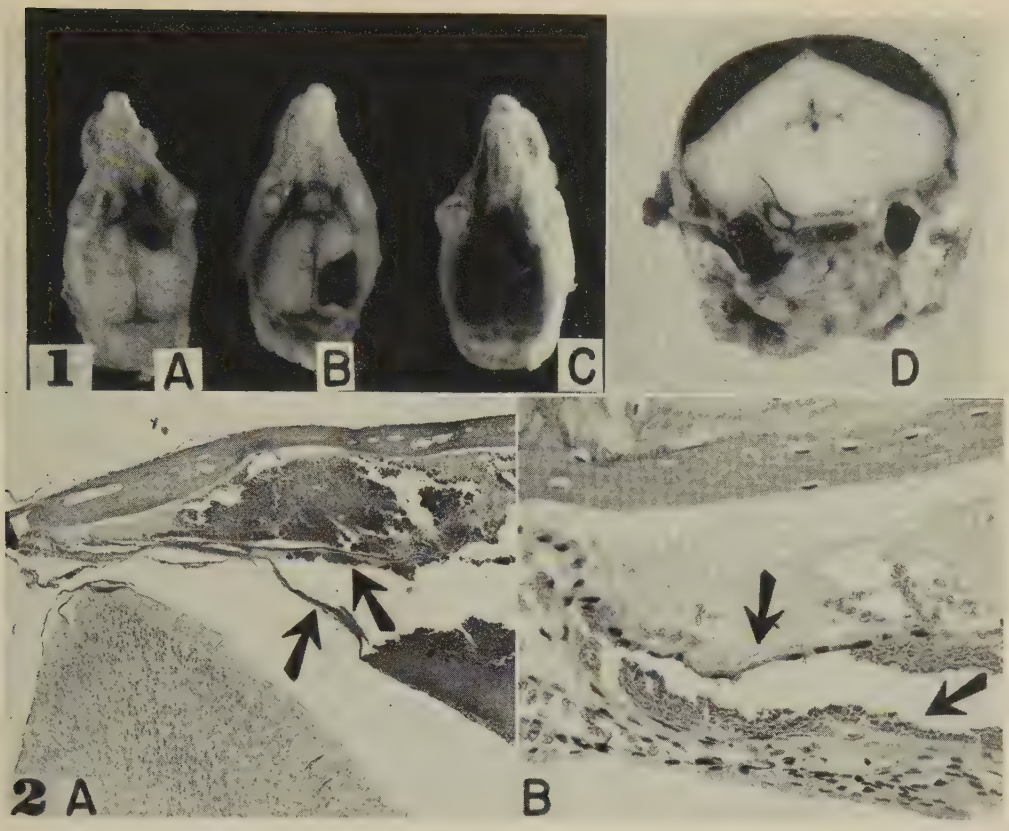

FIG. 1. Representative skulls of rats receiving 20 mg AAN 36 hr before intravenous injection of papain. Animals *A* and *B* were sacrificed at 8 hr; *C* died in 6 hr. *D*. Cross-section of head through parietal bones to show bilateral extradural hemorrhage and brain compression. This animal died 6 hr after papain administration.

FIG. 2 *A*. Low power (× 35) cross-section through parietal bone and underlying brain. Hemorrhage is present between 2 distinct membranes (arrows) which are shown more clearly in *B* (× 300). Here from above downward are shown: bone, periosteal cell layer (arrow) separated from it, and dura (arrow) separated in turn from the periosteal layer.

short time some animals are moribund. The earliest lesion to appear is discoloration over the vertex of the skull. When hair is removed, the discoloration is plainer and seems to be associated with the skull itself. When an animal showing this change is anesthetized and the skin is reflected, widespread hemorrhage is found beneath the cranial bones. The hemorrhage is usually, though not always, bilateral and may extend into the snout region (Fig. 1). The orbit may be involved so that exophthalmos, sometimes bilateral, is seen. Such rats usually die in 6 to 8 hours. Some, as noted below, live. During life hemorrhages have not been noted elsewhere.

At autopsy the extent and effects of hemorrhage beneath the skull are confirmed, and can be determined (Fig. 1D). Moreover, hemorrhage is found in several other characteristic sites: beneath the periosteum of inner surfaces of ribs or about the tibiae and scapulae, and in the orbital areas. Virtually no hemorrhage is found in the viscera. On microscopic examination the exact site of the intracranial hemorrhage can be delineated (Fig. 2). The periosteum, which consists of a single layer of cells, is cleanly separated from the overlying bone by blood cells. In addition, the single layer of periosteal osteoblasts may be found to have been dissected away from the more fibrous dural layer by red blood cells. As a result, 2 distinct membranes lie between the cranial bones and arachnoid-covered brain. Subdural hemorrhage is not

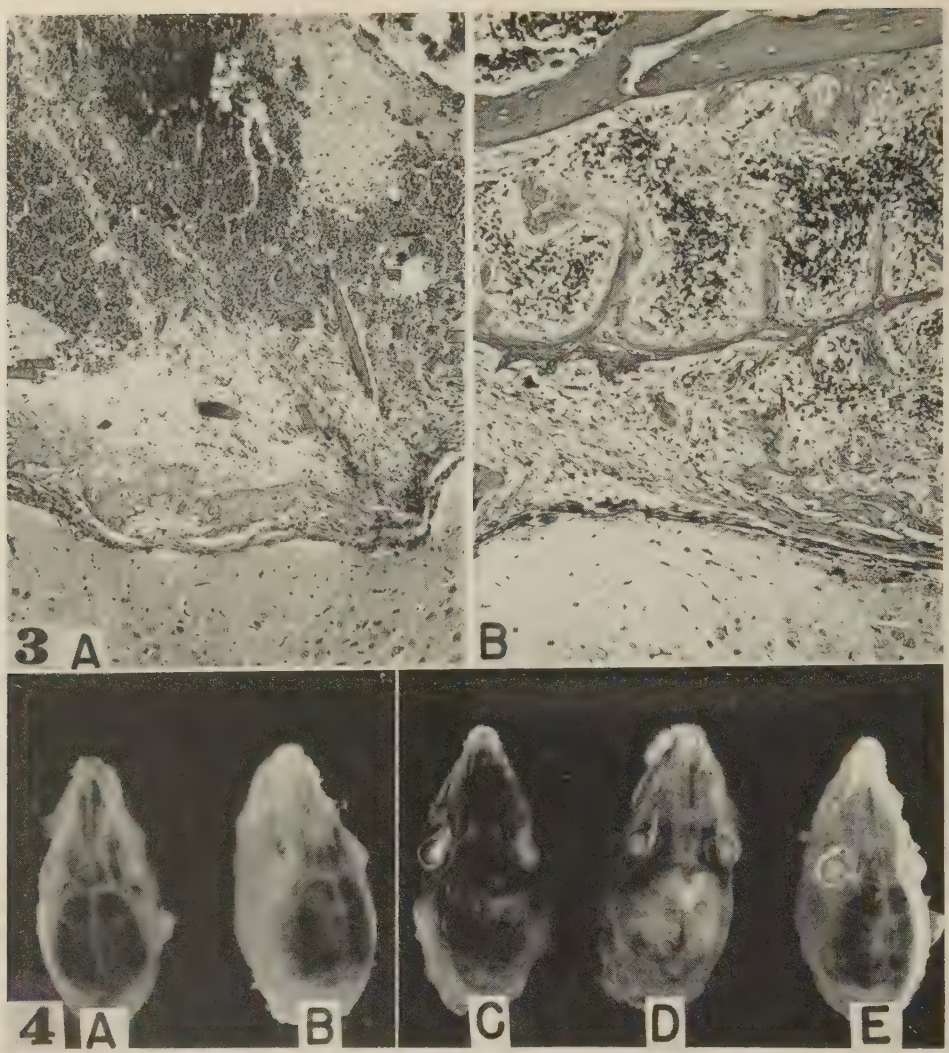

FIG. 3. *A*. Periosteal layer and dura which have been dissected from bone by hemorrhage and are compressing underlying brain (× 100). Lesion is 3 days old. Note bone, including cartilage, which has formed in this time. *B*. Similar area (× 85) from animal in which hemorrhage occurred 5 days previously. Bone formation here is more marked.

FIG. 4. External appearance of skulls of rats which had received 20 mg AAN 24 hr before administration of: *A*, heparin and *B*, polyglucose sulfate. Skulls *C*, *D*, and *E*, are from animals on dicoumarol diet for 36 hr to which AAN had been administered 24 hr before.

present. No hemorrhage is found beneath the external periosteum of the cranium. However, sections through the anterior portion of the skull reveal that hemorrhage is present beneath the periosteum on the outer surface of orbital bone, thus explaining the exophthalmos seen during life. Sections of ribs and long bones reveal separation of the periosteum from their cortices by red blood cells.

If animals do not die of the immediate effects of hemorrhage, the course of organization of the sub-periosteal or extra-dural intracranial hematomata may be followed. Such observations do not help in understanding the pathogenesis of the lesion, but are of interest in that they provide a method for study of new bone formation. The periosteal cells immediately begin to proliferate to form osteoid; this then calcifies, becoming true bone. Moreover, in some cases these same cells appear to

give rise to cartilage which is then replaced by bone (Fig. 3).

When papain is administered in similar doses to animals which have not received preliminary treatment with AAN, no hemorrhage occurs. Larger doses, however, may lead to bleeding, particularly between myocardial fibers. Subperiosteal hemorrhage is not seen. Intravenous administration of papain has been recognized for some time to inhibit blood coagulation(4). The anti-coagulant activity of papain is not clear, though it has been suggested that a heparin-like substance is liberated(5). It seemed possible, therefore, that the hemorrhage produced by papain in animals pretreated with AAN may have been due to the anti-coagulant activity of some material liberated by the enzyme. Consequently, the effects of other anticoagulants have been investigated.

When heparin or polyglucose sulfate, which has a similar anti-coagulant activity(6), are administered to rats which have been treated for one or 2 days with AAN, a syndrome develops which is entirely similar to that just described (Fig. 4). The behavior of rats during life and the gross and microscopic findings at autopsy are identical to those which have been observed following administration of papain. At dose levels administered neither heparin nor polyglucose sulfate leads to spontaneous hemorrhage; however, with larger doses generalized bleeding may be encountered.

Finally, the effect of lowering certain of the essential constituents of the coagulation mechanism has been investigated. Animals were fed dicoumarol in the diet; AAN was administered at varying times thereafter. The results here are complicated by the toxic effects of the drug on growth of rats. If no growth occurs and hence no new matrix is produced, the effects of AAN will be negligible. Hence, the results are not as clear-cut as those produced by papain or the anti-coagulants which have an immediate effect. However, intracranial as well as sub-periosteal hemorrhages have been observed in 8 of a series of 10 animals treated with AAN after being on the dicoumarol-containing diet for 36 hours (Fig. 4).

Discussion. Hemostasis is usually thought to be dependent on the integrity of capillary walls, as well as conditions within and without the lumens of these structures. Normal blood coagulation mechanisms, including platelets, are of importance within vascular lumens. Externally the support of small vessels by connective tissue fibers must be of great importance. The pathogenesis of the hemorrhage encountered can only be speculated upon, since the precise damage to the capillary wall and pericapillary structures must await study with the electron microscope.

An explanation for the lesions which develop as a result of feeding sweet pea seed or chemical agents, such as AAN, has been developed as a result of studies of epiphysial cartilage(1). It is concluded that the defect results from failure of the tropocollagen molecule to polymerize into mature collagen fibrils. In those areas where, normally, new blood vessels are actively proliferating, constant supply of collagen fibers is needed for their support. During the period of AAN administration collagen fibrils presumably are not formed, hence, as capillaries grow they are devoid of their normal collagenous supporting structures. The undersurface of the cranial vault and the subperiosteal areas of long bones and ribs are sites where blood vessels are actively proliferating, perhaps more so than at any other place in the body. Hence, these areas will show the effects most prominently.

When hemorrhages were first encountered in AAN-treated rats following papain administration, we naturally thought that the now well-recognized effect of this enzyme on polysaccharide-protein complexes was likely to be a factor(2). Hence, an alteration of some polysaccharide-protein complex in or about blood vessels with consequent interference with their integrity was considered. Such may be the case. However, we were also aware of the *in vivo* anti-coagulant activity of papain as reported by Kellner, *et al.*(4); moreover, Monkhouse(5) had suggested that heparin-like substances might be liberated by the enzyme. Since papain removes compounds such as chondroitin sulfate A and C from the tissues(2), might not other sulfated polysac-

charides which, in contrast, have anti-coagulant activity, such as chondroitin sulfuric acid B(7) be liberated as well? Such reasoning led us to study the effects of heparin and polyglucose sulfate, in addition to the well-known action of dicoumarol on the coagulation mechanism. The results obtained with these materials clearly involve the blood clotting mechanism, for each produced hemorrhage in AAN-treated animals but by themselves did not. Little would be gained by discussing the mode of action of these materials. The problem is far too complex; in order to elucidate it completely each of the many blood clotting factors which are altered by these agents would have to be investigated. It seems necessary to add that in addition to coagulation factors *per se,* on the clotting mechanism, the role of heparin or dicoumarol on the integrity of the vascular walls themselves must also be kept in mind.

Another outcome of these experiments is of interest to the student of osteogenesis. For as already described, the hemorrhage dissects away the periosteal layer of osteoblasts and hence, affords a means of studying new bone formation by these cells when the effects of the lathyrogenic agent and accessory factors such as heparin or papain have worn off.

Summary. When appropriate amounts of the lathyrogenic agent, AAN, are administered together with papain or the anti-coagulants, heparin or polyglucose sulfate, widespread spontaneous subperiosteal hemorrhages are seen. These do not occur when these materials are administered by themselves. The possible underlying mechanisms of this change are discussed.

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Received July 7, 1959. P.S.E.B.M., 1959, v102.

Tobacco Mosaic Virus Reconstitution Using Inactivated Nucleic Acid.* (25163)

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The conditions required for inactivation of Tobacco Mosaic Virus ribonucleic acid (TMV-RNA) by formaldehyde, nitrous acid and other reagents have been described(1-5). It was clearly shown that in each case the rate of inactivation is considerably greater for the RNA than for the intact virus, and with several reagents first order kinetics of inactivations were observed for the RNA, while the virus appeared to become progressively more resistant. The question then arose whether inactivated RNA could combine with native TMV protein to give stable virus rods. For, such reconstituted inactive virus might be expected to represent a perfect antigen for purposes of immunization against the virus.‡ In contrast to the chemically inactivated virus, the protein of which is of

* Aided by grants from U.S.P.H.S. and National Fn.

† Rockefeller Fn. Postdoctoral Fellow.

‡ The viral protein alone, free from nucleic acid, might be assumed to represent the ideal agent for immunization. However, Aach(6) has recently shown that the dissociated TMV protein differs serologically from intact TMV, each having an antigenic component not shared by the other. Reaggregated protein rods reacted serologically like TMV, but these are not stable under physiological condi-

necessity extensively modified, the reconstituted virus would consist of native unreacted protein encasing a thoroughly modified RNA. The present experiments have shown that rods indistinguishable from TMV are obtained in good yield from propylene-oxide inactivated RNA and TMV-protein. A serological comparison of this inactive material, and its antibody, with active TMV and its antibody showed the 2 virus preparations to be indistinguishable. Other RNA derivatives were also found to reconstitute, giving inactive virus rods with similar or slightly lesser yields than are obtained in infective form from active RNA.

Methods and materials. TMV-RNA was prepared by the detergent method(7), or by means of phenol with versenate buffer (ethylenediamine tetraacetate)(8): the protein by the acetic acid method(9). The reaction with propylene oxide (10% by vol) was performed at 0°, sometimes in the presence of 10^{-4} M pH 7 versenate. After various reaction times the RNA was precipitated and reprecipitated once or twice with 2 volumes of alcohol and .05 ml 3 M sodium acetate/3 ml 67% alcoholic solution. Reconstitutions were carried out at 30° with 0.1% protein and .005% RNA in 0.1 M pH 7.3 pyrophosphate (6 hours). After one low-speed centrifugation, the reconstituted virus in an aliquot of the supernatant was sedimented at 40,000 rpm, redissolved in water, brought to 10^{-3} M with tris-buffer and again sedimented at 40,000. The redissolved pellet was analyzed by UV spectrophotometry (O.D. max 0.27 = 0.01% virus) and at times for phosphorus and arginine content. Infectivity assays were carried out with the RNA prior to and after reconstitution, as well as with the finally separated sedimentable material. The concentration of untreated RNA in test solutions required to obtain about 20 lesions/half leaf is usually 0.1 to 0.5 γ/ml. Thus tests for more than 99.9% inactivation (requiring over 0.5 mg RNA/ml) become progressively more costly and impracticable. However, reconstitution increases sensitivity of test so that 10^{-3} γ/ml RNA are readily detected, and absence of lesions after application of a solution containing 1% reconstituted virus (.5 mg RNA/ml) would signify inactivation by a factor of 10^{-6} to 10^{-7}. For preparation of the antiserum (anti-P.O.-TMV-serum), rabbits were injected with increasing amounts of reconstituted TMV (0.2-1.2 mg/day, prepared from propylene oxide-inactivated RNA) 11 times over a 3 week period, and bled 10 days after last injection. For production of anti-TMV-serum, daily doses of 0.5-2 mg were generally used. The application of light scattering to quantitative evaluation of the virus-antibody reaction will be described elsewhere(10).

Results. TMV-RNA is inactivated by 10% propylene oxide at 0° at the rate of approximately one log/hour (Table I). The yield of 99% inactivated sedimentable material obtainable from this RNA by reconstitution with protein is similar to that obtained from the untreated RNA, and decreases only gradually upon more extensive modification of the RNA. Electromicrographs§ of such material showed only rods of typical diameter; about 30-50% of the mass was usually in the form of rods of 260-300 mμ length.

The interaction of TMV and inactive, reconstituted TMV with their antisera was studied by light scattering technic. The virus preparations were incubated with homologous and heterologous sera in 0.1 M pH 7 potassium phosphate buffer. Concentrations of the reactants were: 38 μg/ml of TMV; 34 μg/ml of inactive reconstituted TMV and 0.1 to 10 μl/ml of antiserum. After incubation at 30° for 3 hours the samples were transferred to the light scattering cell and the Rayleigh ratios at 45°, 90° and 135° (R_{45} and R_{135}) determined. The point of equivalence was obtained from a plot of the dissymmetry ratio, z (R_{45}/R_{135}), against concentration of antiserum. The dissymmetry reaches a maximum value at point of equivalence. Equivalence values obtained were: 1 ml anti-TMV-serum

tions and can, therefore, not be used as antigen. Thus, for purposes of immunization against TMV the RNA is necessary, though only as a stabilizing agent of the rod structure.

§ The authors are greatly indebted to Prof. R. C. Williams and J. Toby for preparation and analysis of the electromicrographs.

TABLE I. Effect of Modification of TMV-RNA on Its Infectivity and Ability to Reconstitute.*

Reagent (conc.)	Reaction conditions	Infectivity, %†	Ability to reconstitute,* %†
Propylene oxide (10%)	0°, pH 7 versenate (.005M) 1 hr	22	94
	Idem 2.5 "	.9	68
	" 24 "	.002	37
	0°, (no buffer, pH 7-8) 1 "	20	71
	Idem 2.5 "	1.3	65
β-Propiolactone (.2%)	0°, 2 h, pH 7 phosphate (.025M)	.2	80
(.5%)	*Idem*	.02	58
Nitrous acid (M $NaNO_2$)	25°, .75 h, pH 4.7 acetate (.25M)	.16	85
	" 3 h " "	<.01	83
U. V. irradiation	0°, .25 h	.7	86
	" 1 h	.015	86
Ribonuclease (10^{-5})‡	25°, .5 h, pH 7 phosphate (10^{-3}M)	<.1	20
(5×10^{-6})	" " "	0–5	0–20
(10^{-6})	" " "	45–57	46–76

* Ability to reconstitute to form virus-like rods with TMV protein in 0.1 M pyrophosphate (Prot/RNA = 20), as measured by ultracentrifuge sedimentation (see *Methods*).

† % of corresponding untreated control.

‡ Enzyme/substrate wt ratio (crystalline pancreatic ribonuclease was used).

= 101 mg inactive reconstituted TMV = 115 mg TMV. 1 ml anti-P.O. – TMV serum = 6.4 mg inactive reconstituted TMV = 7.4 mg TMV. The difference between these values is not significant and the 2 virus preparations were indistinguishable by this technic. The lower titer of the anti-P.O.-TMV-serum is probably due to the lesser concentrations of reconstituted virus used in immunization of the rabbits.

These data seem to show clearly that an anti-TMV serum of high serological activity can be produced with a virus of very low infectivity, obtained by reconstituting propylene-oxide treated RNA with native protein. Inactivation of intact TMV under the same conditions proceeds so slowly that no preparation of similar low infectivity (decrease by over 5 logs) was obtained, but it may be surmised that the extensive alkylation of the protein occurring in the course of the reaction would have affected its serological properties both qualitatively and quantitatively.

RNA inactivated by certain other means (*e.g.* UV light, formaldehyde, nitrous acid, β-propiolactone) also interacted with the native virus protein to give appreciable amounts of sedimentable material (Table I) which can safely be assumed to represent high-specificity viral antigen. In contrast, reactions which decreased the average nucleotide chain length of the RNA, in particular traces of ribonuclease, quickly abolished its ability to form sedimentable material.

It has previously been reported that nucleic acid of a greatly differing strain (HR) gave poor yields upon reconstitution with common TMV-protein, while the opposite combination (HR protein, TMV-RNA) was less disadvantageous(10). We are thus faced with an interesting structural problem: what is believed to be only a different sequential arrangement of the same bases seems to cause enough steric hindrance to interfere with satisfactory RNA-protein interaction while substitution or chemical alteration of bases does not necessarily affect their ability to enter into rod formation. However, it is possible that the extent of chemical modification required for inactivation may be quite low, and therefore localized, while sequential differences of strains may be recurring throughout the chain. Unfortunately no good analytical data for the average extent of interaction of the reagents described are yet available. In a previous study with C^{14} labelled iodoacetate which reacts only slowly with RNA, about 7-10 residues were bound per mole RNA (M.W. 2×10^6, 7000 nucleotides) when over 90% of the infectivity was abolished (36°, 16 hours, pH 8)(11). From the kinetics of inactivation with nitrous acid

Schuster and Schramm(3) concluded that the deamination of 2 groups was, in average, lethal. It thus does seem probable that all RNA inactivating reactions need involve but a small fraction of the potentially reactive sites. The ability to coaggregate with native protein may not be noticeably affected by this extent of modification of the RNA structure.

It seems possible that these observations may have future practical implications. When the nucleic acid free proteins of pathogenic viruses will have been isolated in native form, these may prove more suitable for immunization than the "killed" viruses. If, however, they should prove less than perfect, because they lack the typical virus achitecture‡, then reconstitution of such a native protein with the isolated and thoroughly inactivated preparation of the corresponding RNA might supply the most effective means of safe immunization. Unfortunately, reconstitution has yet been possible only with TMV and its strains.

Summary. TMV-RNA inactivated by various means, in particular by reaction with propylene oxide, retains its ability to form virus-like rods with native TMV-protein. Such reconstituted inactive virus is serologically indistinguishable from the intact virus. The structural and potential practical implications of this observation are discussed.

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Received July 7, 1959. P.S.E.B.M., 1959, v102.

Correlation between Oxygen Consumption and Erythropoiesis in Hypophysectomized Rats Treated with Various Doses of Thyroxin.* (25164)

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Anemia which develops in hypophysectomized animals is accompanied by a decrease in oxygen consumption(1-4). Therapies which have eliminated this anemia, such as a combination of thyroxin, cortisone and growth hormone(3,4), have elevated oxygen consumption. It has been proposed(1,2) that anemia in the hypophysectomized rat is an expression of a decreased need for oxygen of the animal, and that the above therapy proved beneficial because of increased oxygen need resulting from such treatment. This was tested by altering oxygen consumption with various doses of thyroxin given in conjunction with cortisone and growth hormone(4). Oxygen consumption and erythropoiesis varied directly with the dose of thyroxin given. Several investigators studied the effect of thyroxin on hypophysectomized rats(5-8). Partial alleviation of anemia, bone marrow repair and reticulocytosis have all been reported but no attempts were made to relate the effect of various doses of thyroxin to changes in oxygen consumption and alterations in erythropoiesis. If the oxygen need theory is correct, thyroxin

* This investigation supported by a research grant from Nat. Inst. of Arthritis and Metab. Dis., P.H.S.

† With technical assistance of Ellen Sewell.

TABLE I. Effects of Hypophysectomy Alone and Hypophysectomy Followed by Treatment with Varying Doses of Thyroxin on Oxygen Consumption and Total Erythrocyte Volume in Adult Female Sprague-Dawley rats. ± = stand. error.

	Normal controls	Hyp., no treatment		Hyp., 0.01 mg thy./day × 45		Hyp., 0.05 mg thy./day × 25		Hyp., 0.10 mg thy./day × 10	
	32 rats	23 rats	% of normal	14 rats	% of normal	16 rats	% of normal	8 rats	% of normal
Body wt, g	267 ±2.80	213 ±3.73	80	191 ±2.50	72	163 ±2.57	61	163 ±4.8	61
Oxygen consumption, l/m² body surface/hr	9.01 ±.14	6.06 ±.20	67	9.02 ±.25	100	11.17 ±.20	124	9.61 ±.36	107
Plasma vol, cc	8.15 ±.14	6.63 ±.09	81	5.48 ±.07	67	5.96 ±.19	73	5.70 ±.34	70
Blood " "	14.98 ±.20	10.96 ±.14	73	9.54 ±.14	64	11.01 ±.30	73	10.18 ±.55	68
Total erythrocyte vol, cc	6.83 ±.12	4.33 ±.07	63	4.06 ±.08	59	5.05 ±.14	74	4.48 ±.24	66
Idem, per 100 g body wt	2.56 ±.06	2.04 ±.04	80	2.13 ±.05	83	3.11 ±.10	121	2.74 ±.11	107

alone, which increases oxygen need, should stimulate erythropoiesis and one should be able to obtain a variation in erythropoiesis with varying doses of thyroxin in the absence of cortisone and growth hormone. This experiment was devised to determine the effects of various doses of thyroxin on oxygen consumption, and to see whether erythropoiesis would mimic any changes elicited.

Materials and methods. Female rats, 3 to 4 months of age, of Sprague-Dawley (Holtzman) strain were fed standard Purina chow *ad lib.* supplemented once a week with lettuce. Completeness of hypophysectomy was checked at autopsy by examining the organ site with a dissecting microscope. Blood volume measurements were made by the Evans Blue dye method as described by Metcoff and Favour (9). The method used for determining oxygen consumption has been described(1). Bone marrows were saved for histological study. Femurs were removed at autopsy, the ends cut off and one side of shaft cut away. The remaining bone and marrow was placed in Helly fixative, later washed and placed in 70% alcohol. Subsequently the marrow was removed from the bone in a long strip, cleared in dioxane, embedded, sectioned at 6 μ, and stained with Giemsa stain.

Procedure. Ninety-three rats were used; 32 served as normal controls and 61 were hypophysectomized. After the hypophysectomized rats became anemic, they were divided into 4 groups as follows: (1) no treatment, (2) daily subcutaneous injections of 0.01 mg thyroxin,‡ (3) daily subcutaneous injections of 0.05 mg thyroxin, and (4) daily subcutaneous injections of 0.10 mg thyroxin. These doses were chosen because these were the same as given in combination with cortisone and growth hormone(4). Although it was planned to inject all treated groups the same number of days, it was soon apparent that the higher doses were causing a severe weight loss. Accordingly, those receiving 0.01 mg daily were injected for 45 days, those receiving 0.05 mg for 25 days, and those receiving 0.10 mg only 10 days. In spite of this discrepancy in time, the data proved interesting.

Results are summarized in Table I. Animals hypophysectomized and not treated exhibited a 33% decrease in oxygen consumption, accompanied by a 21% decrease in total erythrocyte volume/100 g body weight. Treatment with 0.01, 0.05 and 0.10 mg of thyroxin raised oxygen consumption of the hypophysectomized rats to 100, 124 and 107% of normal respectively; these elevations were accompanied by erythrocyte volumes/100 g body weight 83, 121 and 107% of normal.

Careful study of histological sections of bone marrows (Fig. 1-5) substantiate these results. Hypophysectomy induced the usual

‡ DL thyroxin.

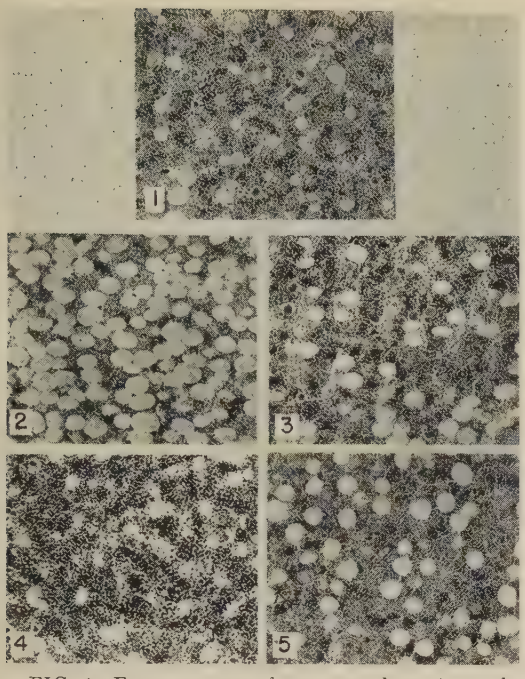

FIG. 1. Femur marrow from normal rat 5 months of age (× 80). Note amount of fat and islands of dark cells representing erythroid elements.

FIG. 2. Femur marrow from hypophysectomized animal 4 months after surgery (× 80). Note increase in amount of fat and decrease in size and distinctness of islands of erythroid elements.

FIG. 3. Femur marrow from rat hypophysectomized for 2 months followed by daily injections of 0.01 mg thyroxin for 45 days (× 80). Note close resemblance to normal marrow above.

FIG. 4. Femur marrow from rat hypophysectomized for 2 months followed by daily injections of 0.05 mg thyroxin for 25 days (× 80). Note hyperplasia with loss of fat and increase in size of islands of erythroid elements.

FIG. 5. Femur marrow from rat hypophysectomized for 2 months followed by daily injections of 0.10 mg thyroxin for 10 days (× 80). Note normal appearance of marrow.

decrease in size and scattering of the islands of erythroid elements and an increase in fat. When slides were thoroughly mixed and treatments of animals unknown, a correct diagnosis of a non-treated hypophysectomized rat was made in all but one marrow. Best results were obtained with daily dose of 0.05 mg of thyroxin; the marrows in these animals were stimulated to a degree that islands of erythroid elements were larger than those found in normal controls and fat was decreased in amount when compared to normal. All rats were diagnosed as either normal or stimulated beyond normal. Treatment with 0.10 mg of thyroxin induced a marrow with normal appearance of erythroid elements and approximately a normal amount of fat. All but one of these marrows were diagnosed as normal. Treatment with the lowest dose, 0.01 mg, induced variable results. They were diagnosed as normal, containing too much fat, or questionable.

Discussion. Decreased oxygen need has been proposed as a cause for anemia which develops in hypophysectomized animals. This theory is supported by the fact that oxygen consumption is decreased after hypophysectomy, and that this low oxygen consumption is maintained even after the erythrocyte picture is returned to normal by transfusions(1). Therapeutic measures (except cobalt) proved effective in eliminating post-hypophysectomy anemia, have also elevated oxygen consumption; those found ineffective have not(2). This theory finds further support when one considers that the erythrocyte level after hypophysectomy does not continue to fall but plateaus at a fairly constant level 30 to 40 days after hypophysectomy. In addition, marrow in the hypophysectomized rat will respond normally under conditions of decreased oxygen tension(10), or if made more anemic by bleeding or by total body irradiation(11-14). Erythropoiesis does occur in hypophysectomized animals when the level of erythrocytes is lowered below the number actually needed by that animal.

In our experiment, injections of various doses of thyroxin into hypophysectomized rats induced increases in oxygen consumption which were accompanied in animals receiving the 2 highest doses by similar changes in total erythrocyte volume/100 g body weight. Increased erythropoiesis in bone marrows was observed in these groups. Similar results were reported(4) when cortisone and growth hormone were given in combination with the same doses of thyroxin. The 2 experiments differ with respect to response to lowest dose of thyroxin. Oxygen consumption was returned to normal in both instances, but the erythrocyte picture was normal only when thyroxin was combined with cortisone and growth hormone. However, it is apparent that when one increases the oxygen demand beyond this low-

est level the relationship between oxygen consumption and erythropoiesis can be demonstrated without cortisone and growth hormone. This correlation of oxygen consumption and erythropoiesis with thyroxin alone provides another instance in support of the oxygen need theory.

The marked loss of body fat in our experiment must be taken into consideration. There is certainly no doubt that body size must be considered when studying blood volumes as well as when determining oxygen consumption. It has been suggested that this loss of fat may not be similar to loss of a combination of all tissues and that the expression of total erythrocyte volumes/100 g body weight is not correct. The fact that these findings in total erythrocyte volumes/100 g body weight were accompanied by similar changes in marrow activity would seem to indicate that our conclusions are valid.

One cannot conclude from this experiment that post-hypophysectomy anemia is caused by a simple hypothyroidism in that the anemia of thyroidectomy is less severe than that of hypophysectomy. Loss of growth and adrenocorticotrophic hormones is also involved. Animals receiving our combined thyroxin-cortisone-growth hormone therapy are in much better health, body weight and growth are maintained and they are in better hematological condition than those receiving the same doses of thyroxin alone. What role these hormones play in the total process is unknown. It cannot be definitely determined whether growth hormone and cortisone act directly on the marrow or *via* some other mechanism. Evans *et al.*(15,16) have shown that there is a synergistic action of thyroxin and growth hormone on calorigenesis.

Summary. Hypophysectomized rats were treated with 0.01, 0.05 or 0.10 mg of thyroxin daily. Oxygen consumption in the 3 groups of animals was 100, 124 and 107% of normal; total erythrocyte volumes/100 g body weight were 83, 121 and 107% of normal. Hypophysectomized controls exhibited oxygen consumption 67% of normal with total erythrocyte volume/100 g body weight, 79% of normal. These data support the oxygen need theory of post-hypophysectomy anemia.

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Received July 7, 1959. P.S.E.B.M., 1959, v102.

Isolation and Purification of Streptococcal Hyaluronic Acid. (25165)

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Since the first isolation of hyaluronic acid from strains of Groups A and C streptococci (1) numerous methods for extraction and purification of streptococcal hyaluronic acid have been reported(2-4). In view of possible significance of bacterial hyaluronic acid and its depolymerization products in certain physiological and medical problems(5-7), it was of interest to investigate procedures whereby large quantities of streptococcal hyaluronic acid may be prepared conveniently for these studies. Present methods for preparation and production of bacterial hyaluronic acid depend mainly on growth of the organism in complex organic culture media many of which contain a polysaccharide hydrolyzed by hyaluronidase(8,9). Moreover, purification technics are laborious, complicated and feasible for isolation of relatively small quantities of material. Our purpose is to present a simple semi-synthetic medium, developed with the purpose of providing conditions not only for optimum growth of *S. pyogenes* but also for formation of large quantities of hyaluronic acid. A rapid, reproducible method for isolation of partially purified hyaluronic acid from the filtrate of Group A streptococcus will also be described.

Materials and methods. Culturing methods. Streptococcus pyogenes Group A type 9 (T9/63/3), obtained from the Hospital of Rockefeller Institute, was used. The organism was maintained on blood agar containing 5% defibrinated sheep's blood. Actively growing seed cultures were prepared by transfer of growth from 24 hour stock slants to 10 ml of culture medium (described below) before transfer to 500 ml of similar broth which was then incubated for 18 hours at 37°C. The medium used consisted of casein hydrolysate (enzymatic) 50 g; $Na_2HPO_4 \cdot 12\ H_2O$, 11.2 g; KH_2PO_4, 0.96 g; $MgSO_4$, 0.04 g; Ca-d-pantothenate, 1 mg; pyridoxine HCl, 1 mg; riboflavin, 0.1 mg; distilled water, 1 liter. The medium was adjusted to pH 7.6-7.8 with 5N NaOH and sterilized by autoclaving at 120°C for 20 minutes. After sterilization, the medium was supplemented with glucose (sterilized by filtration through Seitz filter) to a final concentration of 10 mg/ml. Nine-liter Pyrex carboys containing 5 liters of medium were employed. Inoculation was made with 500 ml of seeding culture/5 liters medium. *Production of hyaluronic acid.* Hyaluronic acid was readily produced by this strain of streptococcus after 16-18 hours incubation at 37°C. The contents of the 9-liter carboys was heated in boiling-water bath for 1 hour, quickly cooled and formalin added with mixing to a final concentration of 2%. The bottles were stoppered with rubber stoppers and stored at room temperature. Crude hyaluronic acid broths treated in this manner may be stored at room temperature for at least 6 months with no loss in yield of final product. The crude broth, clarified by filtration through "Hyflo Super-Cel" (Johns Manville) was treated with 3 volumes of cold acetone. The highly viscous brown liquid phase which immediately separated was stored overnight in cold room (5°C). The supernatant fluid was decanted and the brown liquid phase was diluted with equal volume of distilled water. To 1 liter of viscous brown liquid phase extract, 250 ml of glacial acetic acid and 125 g of potassium acetate were added. The mixture was stirred 5 minutes and poured into 1,250 ml of cold 95% ethyl alcohol producing a white flocculent precipitate. After 30 minutes in ice bath, the precipitate was collected by filtration with suction through "Hyflo Super-Cel" and dissolved with distilled water. A second and third reprecipitation was performed in this manner. The final precipitate was washed with absolute ethyl alcohol, dissolved in distilled water, dialyzed against frequent changes of distilled water for 48 hr at 4°C and then freeze-dried. The yield of potassium hyaluronate from 5 liters of culture medium averaged 1.2 g. *Chemical analysis.* The purified

product gives negative tests for glycogen and sulfur. Nitrogen was determined by micro-Kjeldahl method on 35 preparations and values varied between 2.9 and 3.9%, average of 3.4%. Glucosamine was determined by the method of Elson and Morgan(10) as modified by Rimington(11). Glucosamine content for the mucopolysaccharide was 32.3%.*

Results. Rate of hyaluronic acid formation in broth. It has been demonstrated that streptococci which produce hyaluronic acid also elaborate hyaluronidase(12). Thus coexistence of the enzyme and its substrate in the same culture is apparently responsible for disappearance of hyaluronic acid from the broth culture on continued incubation. If this reasoning holds true, prevention of the reaction between enzyme and substrate ought to be possible by addition to the broth of a hyaluronidase inhibitor. Under these circumstances the inhibitor might prevent disappearance of hyaluronic acid in the broth culture that is otherwise certain to occur after the logarithmic growth phase ceases. It was, therefore, of interest to correlate hyaluronic acid production with age of organism as well as to determine the effect of hyaluronidase inhibitor on rate of hyaluronic acid formation in broth. An alginic acid sulfate preparation, previously found very effective inhibitor of hyaluronidase (unpublished observations), was used. Nine-liter carboys containing 5 liters of semi-synthetic stock medium with added alginic acid sulfate (0.1 mg/ml) were inoculated and incubated as described. Aliquots were removed at varying intervals and assayed for hyaluronic acid. Technics of sampling and measurement of hyaluronic acid concentration have been reported(13). The results shown in Fig. 1 demonstrated that rate of hyaluronic acid synthesis in casein hydrolysate broth without addition of alginic acid sulfate is maximal after first 18 hours of incubation. Continued incubation revealed a marked decrease in hyaluronic acid originally produced, resulting in its complete disappearance in 64 hours. However, hyaluronic acid levels in broth culture of *S. pyogenes* containing 0.1

* We are indebted to Dr. Gordon Ellis, Wyeth Inst. for Med. Research for these data.

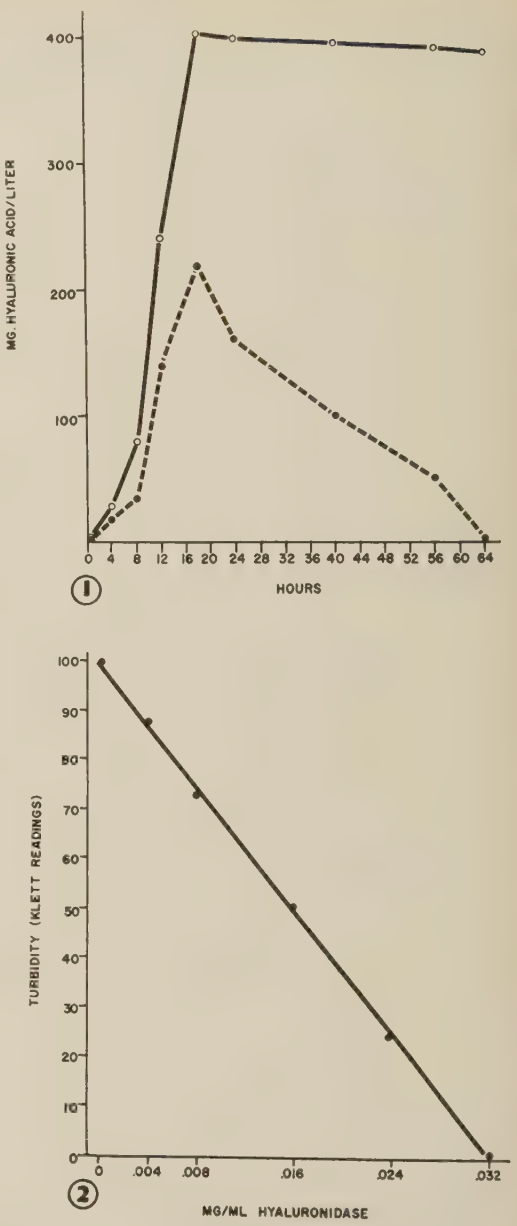

FIG. 1. Concentration of hyaluronic acid in growing culture of Group A *S. pyogenes* in semi-synthetic culture medium. ●—● = hyaluronic acid formed in absence of hyaluronidase inhibitor. ○—○ = hyaluronic acid formed in presence of hyaluronidase inhibitor.

FIG. 2. Depolymerization of partially purified streptococcal hyaluronic acid by purified bovine testicular hyaluronidase. The system consisted of 0.5 ml of 0.4 mg/ml hyaluronic acid and 0.5 ml of various dilutions of hyaluronidase. Incubated 30 min. at 37°C. Reaction stopped and turbidity developed by addition of 4 ml of 1:40 acidified horse serum, pH 3.1. Read in Klett-Summerson colorimeter; red filter No. 66.

mg/ml of alginic acid sulfate were stabilized on continued incubation and a significant increase in hyaluronic acid levels of broths containing hyaluronidase inhibitor was obtained. The curves (Fig. 1), are typical of those obtained with the mucopolysaccharide and are readily reproducible with every batch of material.

Depolymerization of purified hyaluronic acid by hyaluronidase. Depolymerization of partially purified streptococcal hyaluronic acid by purified bovine testicular hyaluronidase (1550 USP units/mg) was studied using turbidimetric assay method described previously(14). A turbidity value of 100 scale divisions on the Klett-Summerson colorimeter was produced by interaction of 0.2 mg of polysaccharide and acidified horse serum. A linear correlation was found between enzyme concentration and depolymerization of the polysaccharide substrate (Fig. 2).

Summary. 1. Hyaluronic acid was released from cells of a Group A *Streptococcus pyogenes* when the latter was grown in medium in which all constituents except casein hydrolysate were chemically defined. With this medium it has been possible to produce high yields of streptococcal hyaluronic acid following 16 to 18 hour incubation at 37°C. 2. Stability of hyaluronic acid in growing cultures of *S. pyogenes* following addition of a hyaluronidase inhibitor, alginic acid sulfate, to the medium, together with a significant increase in hyaluronic acid levels of the broths is further evidence that strains of streptococci which produce hyaluronic acid also produce hyaluronidase. 3. Isolation and partial purification of the bacterial mucopolysaccharide are described.

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Received July 9, 1959. P.S.E.B.M., 1959, v102.

Induction and Maintenance of Lactation in Rats by Electrical Stimulation of Uterine Cervix.*† (25166)

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Electrical stimulation of the uterine cervix can induce pseudopregnancy in sexually mature rats(1,2) and hasten sexual maturation in prepubertal rats(3). Presumably, these are elicited by nervous stimuli through sympathetic and hypothalamic pathways, resulting in release of FSH, LH and luteotropin by the anterior pituitary(2,3). There is also evidence that mechanical stimulation of the uterine cervix may elicit oxytocin and possibly vasopressin release from the posterior pituitary(4,5). These findings, as well as our recent observation that epinephrine, acetylcholine or serotonin can initiate and maintain

* Published with approval of Mich. Agr. Exp. Sta. as Jour. Art. No. 2469.

† This work was supported in part by USPHS Grant to senior author.

lactation in rats(6,7), has prompted us to determine whether electrical stimulation of the rat uterine cervix can liberate sufficient prolactin from the anterior pituitary to initiate or maintain lactation. It was also of interest to see whether similar effects on lactation could be elicited by oxytocin or vasopressin.

Methods. Mature virgin female rats (Carworth) weighing between 200-250 g were injected subcutaneously with 10 μg estradiol daily for 10 days to produce mammary development. This treatment has previously been shown to induce limited but definite lobulo alveolar growth(7). For the next 5 days, groups of 5 rats each were treated as follows: controls, physiological saline once daily; 1 IU oxytocin‡ twice daily; 1 IU vasopressin‡ twice daily; electrical stimulation of uterine cervix twice daily; *ibid,* 3 times daily; 70 mg morphine sulfate/kg BW twice daily, followed 20-30 minutes later by cervical stimulation. Electrical stimulation was conveyed to the cervix by platinum electrodes which were insulated except for the tips. A low current was supplied by an Electrodyne Stimulator set at a frequency of 20 cycles/second for 30 seconds. The current was just detectable on our finger tips and usually produced only momentary struggling by the rats. Post-partum rats were used to determine effects of cervical stimulation on maintenance of lactation. Litters were removed on 4th day after parturition and groups of 5 mother rats each were treated for 10 days as follows: controls, physiological saline; electrical stimulation of cervix twice daily; 1 IU oxytocin twice daily; 1 IU vasopressin twice daily. All rats were killed on day after last treatment, and the right inguinal mammary glands were removed for histological examination. The glands were fixed in Bouin's fluid, sectioned at 6 μ and stained with eosin and hematoxylin. Each gland was examined microscopically for degree of secretion and mammary development. Ovaries, uterus, adrenals and thymus glands were also removed and weighed.

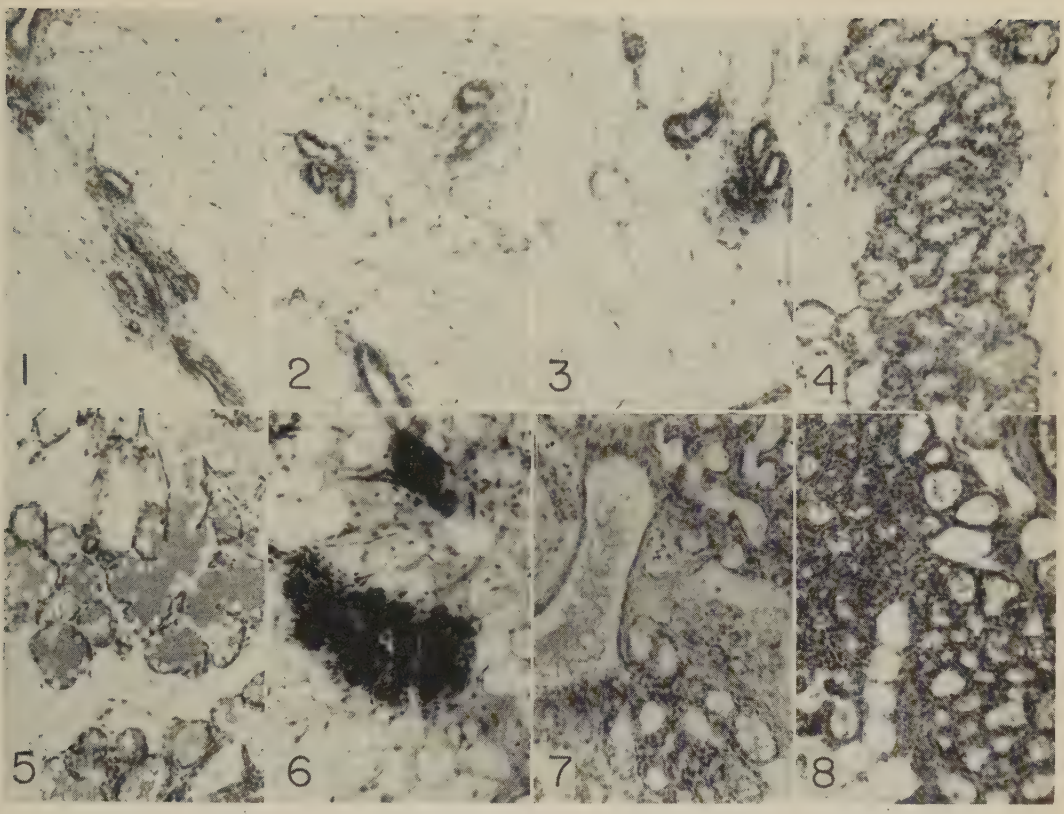
Results. In virgin rats, injections of saline (Fig. 1), oxytocin (Fig. 2) or vasopressin (Fig. 3) resulted in mammary regression to practically a bare duct system and no secretion. In sharp contrast, 4 out of 5 rats in each of the 2 groups electrically stimulated 2 or 3 times daily, showed definite secretion and intact lobulo alveolar structure (Fig. 4). The 5 rats given morphine sulfate prior to electrical stimulation all showed intense mammary secretion (Fig. 5), equivalent to that usually seen in lactating rats after parturition.

In postpartum rats, controls injected with saline for 10 days after litter removal showed pronounced mammary involution and no secretion (Fig. 6), while animals electrically stimulated (Fig. 7) or given oxytocin (Fig. 8) showed moderate secretory activity and retardation of mammary involution. Vasopressin was only slightly effective in these respects. None of the treatments produced significant changes in weights of ovaries, uterus, adrenals or thymus glands, as compared with saline-injected controls.

Discussion. These results demonstrate that electrical stimulation of rat uterine cervix can initiate milk secretion after development of mammary glands with estradiol, and can maintain secretory function and retard mammary involution in postpartum rats after litter removal. These effects are believed to be mediated through release of prolactin from the anterior pituitary *via* the autonomic nervous system and hypothalamus. Evidence for participation of the autonomic nervous system is provided by the following: (a) Interruption of sympathetic pathways during electrical stimulation of the uterine cervix prevents pseudopregnancy(2) or hastening of sexual maturation(3) in rats. (b) Dibenamine or atropine injections may inhibit prolactin liberation from the AP of lactating rats(8). (c) Epinephrine, acetylcholine or serotonin can initiate or maintain lactation in rats with developed mammary glands(6,7).

Although morphine has been reported to inhibit ACTH release(9), it did not prevent and appeared to enhance mammary response to electrical stimulation of the cervix. This cannot be considered conclusive however, since further work has indicated that the dose of morphine sulfate used failed to evoke charac-

‡ Oxytocin and vasopressin were supplied through courtesy of Dr. D. A. McGinty, Parke, Davis and Co.



FIGS. 1-5. Mammary sections from virgin rats given estradiol for 10 days, followed for 5 days by (1) saline, (2) oxytocin, (3) vasopressin, (4) electrical stimulation of uterine cervix 3 times daily, (5) morphine sulfate and electrical stimulation of uterine cervix 2 times daily.

FIGS. 6-8. From postpartum rats, treated for 10 days after litter removal with (6) saline, (7) electrical stimulation of uterine cervix, (8) oxytocin.

teristic symptoms in the rat after the first 3 days of injection.§ Apparently the lactation-inducing effects of electrical stimulation of the cervix were not mediated to any significant extent through ovaries or adrenals, since neither the weights of these organs nor of the uterus and thymus were altered. However, in view of previous demonstrations that manipulation of the uterine cervix stimulates liberation of gonadotropic hormones(1-3), the possibility cannot be excluded that slight alterations occurred in ovarian or adrenal cortical activity, not detectable by changes in organ weight.

Neither oxytocin nor vasopressin initiated lactation in these rats. Oxytocin has previously been shown to be incapable of initiating lactation in rabbits with developed mammary glands(10,11). Mammary secretion was maintained in postpartum rats by oxytocin and to a much lesser extent by vasopressin, confirming previous reports which formed the basis for the claim that oxytocin stimulates prolactin release from the anterior pituitary (11-13). These workers also believe that oxytocin is responsible for releasing prolactin when the nipples are stimulated by suckling during lactation. However, simultaneous release of oxytocin and prolactin, which occurs during stimulation of uterine cervix or nipples, may be effected by independent mechanisms, rather than by assuming that oxytocin is responsible for prolactin liberation. This is supported by observations that (a) suckling by litters results in rapid discharge of pituitary prolactin content in mother rats(14,15), whereas injections of oxytocin or posterior

§ We have recently found that injections of morphine sulfate alone can initiate lactation in estrogen-primed rats.

pituitary extract fail to induce a similar decrease in pituitary prolactin in the rat, guinea pig or rabbit(15,16), (b) injections of oxytocin and prolactin together into postpartum rats after litter removal are more effective in maintaining lactation than either hormone alone(17), (c) injections of reserpine into lactating rats inhibits oxytocin release but stimulates prolactin discharge from the pituitary(18).

Our recent work suggests that the effectiveness of oxytocin in maintaining mammary secretion in postpartum rats after litter removal depends upon removal of accumulated secretion from the alveolar lumina and smaller ducts, thereby permitting further synthesis of milk by the alveoli(19). Histological preparations of mammary tissue from postpartum rats after litter removal have shown that a single injection of oxytocin, following treatment with prolactin for 5 or 10 days, produces ejection of accumulated secretion from the alveolar lumina and smaller ducts within 10 minutes. The secretory material is believed to enter the larger ducts and numerous stromal tissue spaces, from which it can gradually be reabsorbed into the circulation or perhaps be partially reutilized for milk synthesis. Details of these observations will be published.

Passage of the fetus through the uterine cervix at parturition may provide a stimulus for prolactin release and initiation of lactation at this time. This could supplement the stimulus to prolactin secretion believed to come from estrogen at the end of pregnancy(10,11,16). However, cervical stimulation by fetal passage is probably not essential for lactation, since prolongation of gestation by progesterone(20, 21) or removal of the entire uterus during latter part of pregnancy(16) do not prevent initiation of milk secretion. We have previously suggested that stimuli arising from mammary glands at end of gestation may also evoke prolactin release and help to initiate lactation(22, 23). This problem is under further investigation.

Summary. 1. The uterine cervix of virgin, sexually mature rats was stimulated electrically 2 or 3 times daily for 5 days, following daily injections of 10 μg estradiol for 10 days to develop the mammary glands. Milk secretion was initiated in 8 out of 10 rats by this treatment, whereas 5 saline-injected controls showed no secretion and mammary involution. Twice daily injections of 70 mg morphine sulfate/kg BW prior to uterine stimulation did not inhibit lactation in 5 rats, and it was actually more intense in these animals. Twice daily injections of 1 IU oxytocin or 1 IU vasopressin for 5 days failed to initiate mammary secretion, showing that these hormones are not responsible for prolactin release in the cervical-stimulated rats. 2. Following litter removal on 4th postpartum day, injections of saline into mother rats for 10 days resulted in cessation of lactation and pronounced mammary involution. Twice daily electrical stimulation of the uterine cervix or injections of oxytocin for 10 days maintained secretory activity and retarded mammary involution, whereas vasopressin was much less effective in these respects. 3. It is concluded that electrical stimulation of the uterine cervix initiates lactation through sympathetic and hypothalamic pathways, resulting in prolactin release from the anterior pituitary. Oxytocin and to a lesser extent, vasopressin, are believed to maintain mammary secretion in postpartum, non-suckled rats by ejecting accumulated secretory material from the alveolar lumina and smaller ducts into the larger ducts and stromal tissue spaces, where it can be readily absorbed into the circulation.

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Received July 13, 1959. P.S.E.B.M., 1959, v102.

Metabolism of Sulfobromophthalein in Hepatectomized and Hepatectomized-Nephrectomized Dog.* (25167)

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Sulfobromophthalein (BSP), because it is preferentially removed by the liver, has been used extensively as a liver function test in clinical medicine. Recent studies have shown that BSP is metabolized by the liver(1-5) and excreted into the bile, in part at least, as a mercaptide with cysteine and possibly with a compound containing cysteine, glycine and glutamic acid(6). Previous studies of BSP metabolites were carried out in normal human subjects and animals(2-4), in isolated perfused livers(3) and in patients with liver disease(7). In this investigation the ability of tissues other than the liver to metabolize BSP was studied in hepatectomized and hepatectomized-nephrectomized dogs.

Methods. Two normal male dogs, weighing 16 and 23 kg respectively, were hepatectomized under pentobarbital anesthesia. The liver, isolated by the method of Dakin *et al.* (8), was removed, along with a short segment of the inferior vena cava. The resulting gap in the remaining inferior vena cava was bridged by a plastic catheter. Animals were maintained by continuous infusion of 20% glucose. The blood volume was restored when necessary by transfusion of dog blood. Control samples of urine and blood were collected. The animals were then given commercial BSP intravenously (5 mg/kg of body weight). Blood samples, taken at timed intervals thereafter, were analyzed for total BSP and BSP conjugates. Urine, collected from each animal during a 2 hr period after injection of BSP, was also analyzed for total and conjugated dye. Two additional dogs were hepatectomized and also nephrectomized. Fifteen to 30 min. later, control blood samples were obtained. Commercial BSP was then administered intravenously (5 mg/kg of body weight). Blood specimens were taken at timed intervals and analyzed for total and conjugated BSP. Concentrations of total BSP in serum and urine were determined by the method of Gaebler(9). To quantitate the proportion of conjugate, measured aliquots of serum and concentrated urine were extracted with acetone (1:3 v/v). The resulting supernatants were dried under vacuum. The material was taken up in small aliquots of water and chromatographed on Whatman 3 MM pa-

* Supported by grants from U.S.P.H.S.

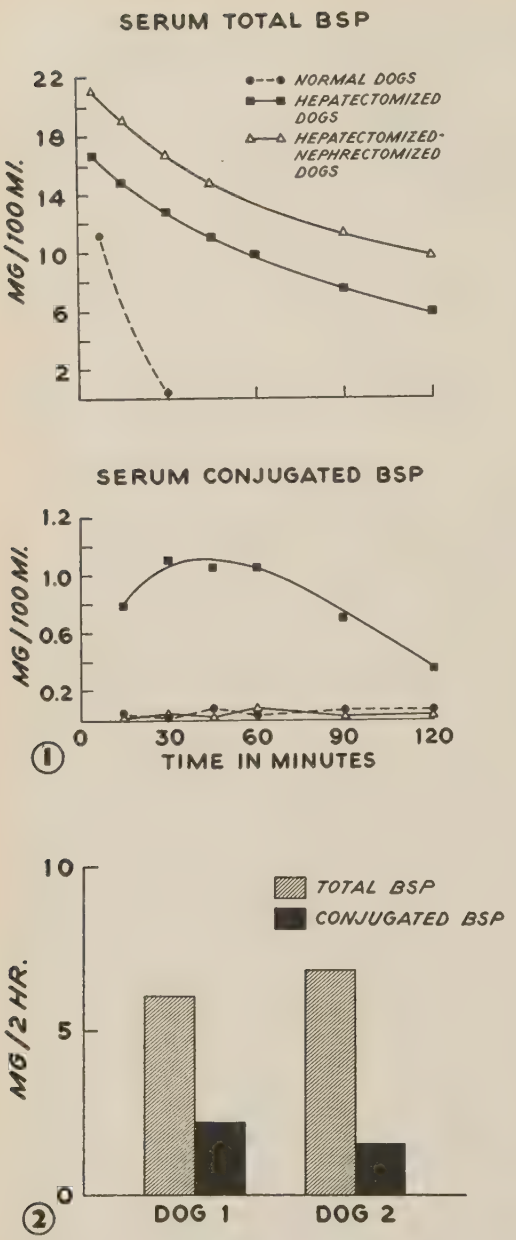

FIG. 1. Concentrations of BSP and BSP metabolites in serum after intravenous administration of dye into normal and surgically treated dogs.

FIG. 2. Excretion of BSP and its conjugates in urine of hepatectomized dogs.

per in an ascending system employing *t*-butanol/water (1.73:1 v/v) for 6 hr. BSP bands were developed by spraying them with freshly prepared 0.12 N NaOH in 95% ethanol. The metabolite bands (Rf approximately 0.65 and 0.23) were cut from the strip, pooled and macerated in 25 ml of 0.05 N NaOH. The mixture was centrifuged for 10 min. to remove the paper pulp. Samples of 6 ml of supernatant were read against a blank of 0.05 NaOH at 565 and 620 mμ. The unconjugated band (Rf approximately 0.88) was similarly extracted from the paper and quantitated. Standard deviation obtained after duplicate determinations on 15 different serum samples was 2.1%(7).

Results. Normal dogs. In 2 normal intact dogs, BSP was cleared rapidly (Fig. 1). After 45 min. only 0.36 and 0.13 mg/100 ml of the dye remained in circulation. Only traces of the BSP metabolites were observed. No appreciable quantities of BSP appeared in the urine of these animals.

Hepatectomized dogs. In hepatectomized dogs BSP was removed from the blood more slowly than in normal animals, but still at a significant rate (Fig. 1). Within 45 min. 35% of the dye present 7 min. after injection had been removed; by 2 hr. 65% had been cleared. During this interval the level of BSP metabolites rose to about 10% of total circulating pigment at 45 min.; thereafter it gradually decreased. After 2 hr. only about 10% of total BSP cleared could be accounted for in the urine. Approximately one-fourth to one-third of the urinary BSP, however, was in the conjugated form (Fig. 2).

Hepatectomized-nephrectomized dogs. Rate of disappearance of BSP from the blood of hepatectomized-nephrectomized dogs was similar to that of the hepatectomized animals (Fig. 1). Only trace amounts (less than 2%) of BSP metabolites, however, were found in the blood of the hepatectomized-nephrectomized dogs. These trace amounts were too small to measure quantitatively and technically were of questionable significance.

To demonstrate that the BSP metabolite bands did not arise as artifacts of chromatography or from nonspecific complex formation in blood and urine, specimens of serum and urine from both pairs of animals were incubated with commercial BSP for 1 hr. at 37°C and chromatographed. No significant levels of material corresponding in mobility to the BSP conjugates were found.

Discussion. Rapid removal of BSP from

the blood of the hepatectomized dog confirms the observation of Cohn *et al.*(10) in similarly treated animals that large amounts of the dye are removed from the blood during the first hour after injection. The appearance of significant quantities of BSP metabolites in the serum of these animals indicates that extrahepatic conjugation of BSP can occur. Since a higher percentage of metabolites was found in the urine than in the serum, the kidney apparently can either a) synthesize or b) preferentially excrete BSP metabolites. The almost complete absence of metabolites in the serum of the hepatectomized-nephrectomized animals favors the first possibility. The ability of the kidney to conjugate pigment has been demonstrated previously in the case of bilirubin(11). Furthermore, Mills and Wood (12) have shown that kidney slices contain the conjugative enzymes that may be required for BSP mercaptide synthesis.

Although the 2 hepatectomized dogs cleared 65% (52 and 75 mg) of injected BSP in 2 hr., only about 10% of the cleared dye appeared in the urine. Apparently the major portion of the dye in the hepatectomized animal is removed by uptake in peripheral extrarenal tissues. This conclusion is further supported by the fact that in the hepatectomized and the hepatectomized-nephrectomized dogs BSP was cleared from the blood at similar rates. The differences between the initial blood levels of BSP in the 2 pairs of animals may be attributable in part to differences in blood volume due to losses during surgical procedure or to rapid concentration of a portion of the pigment by the kidney in the animals which had only been hepatectomized.

A recent study(7) has shown that significant amounts of serum BSP conjugates are found in diseases of the liver and the biliary tract. In the normal organism in which BSP is rapidly removed by the liver, extrahepatic uptake and conjugation may play only a minor role. In severe liver disease, however, significant removal and conjugation of BSP by extrahepatic tissue may conceivably take place.

Summary. 1. Conjugation and rate of removal of sulfobromophthalein from the blood stream were studied in hepatectomized and hepatectomized-nephrectomized dogs. 2. Clearance of BSP from blood of hepatectomized dogs occurred at a slow but significant rate. Only 10% of cleared dye was excreted in urine. Conjugates of BSP appeared in serum and in greater percentage in urine. 3. Hepatectomized dogs after nephrectomy still cleared BSP at significant rate but had only questionable traces of metabolites in the serum. 4. It was concluded that the kidney is capable of conjugating BSP but that most of the dye clearance in the hepatectomized dog can be attributed to extrarenal mechanisms in the peripheral tissues.

Surgical procedures were carried out by Drs. F. William Heer and Robert S. Seipel, Univ. of California School of Med., San Francisco.

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Received July 13, 1959. P.S.E.B.M., 1959, v102.

A Mode of Action for Thyroid Inhibition by Reserpine.* (25168)

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Reserpine administration has been reported to depress O_2 consumption in rats(1) and guinea pigs(2). Other studies have shown reserpine to decrease thyroidal-I^{131} uptake(3, 4), release rate(5) and thyroxine secretion rate(5). Little is known, however, of the mechanism by which reserpine exerts its antithyroid activity. It has been suggested the drug inhibits thyroid function by 1) inactivation of thyroxine(1,2) or thyrotropin (TSH), 2) inhibition of organic-binding I_2(3,4) or 3) suppression of TSH secretion through inhibition of hypothalamic centers(5,6). In the present study, we attempted to determine which of the suggested mechanisms is responsible for the antithyroid activity of reserpine in the rat.

Materials and methods. Young mature female rats of Sprague-Dawley-Rolfsmeyer strain weighing 190-230 g were housed in a room artificially illuminated during daylight hours and at uniform temperature of 79 ± 1°F. They were allowed free access to Purina Lab Chow and water. *Exp. 1.* Fifteen rats were injected i.p. with 2 μc carrier-free I^{131}. Forty-eight hours were allowed for I^{131} fixation by thyroid gland. External neck counts were made at this time and every 24 hours thereafter by first anesthetizing each animal with ether, then placing it on a lead plate with neck resting on a scintillation probe containing a 2″ NaI crystal. Care was taken in placement of the animal to insure the same geometrical relationship at each successive counting period. Thyroidal radioactivity was measured with scintillation counter, Nuclear-Chicago (N.C.) Model DS5, connected to rate meter, N.C. Model 1620A. Conventional corrections were made for background and radioactive decay. Neck counts were made on days 2-4 after initial count to establish that thyroidal-I^{131} output was proceeding normally. Each rat received daily subc. injections of 2.5 μg/100 g l-thyroxine, to block endogenous TSH secretion, .2 unit TSH‡ and 10 μg/100 g reserpine beginning on days 4, 7 and 10, respectively, after initial count and continuing throughout experimental period. Thyroidal-I^{131} release curve was constructed by plotting average percent dose against time in days. Average hourly release thyroidal-I^{131} (k′4) was calculated by the method of Brownell(7). *Exp. 2.* Fifty-nine additional female rats were injected daily with 10 μg/100 g reserpine and/or 4 mg/100 g 1-methyl-2-mercaptoimidazole (tapazole) for 10 days. Sixteen served as controls of which half were injected with .1 ml/100 g distilled water/day. Body weight of each rat was recorded daily. Twenty-four hours after last injection, each animal was weighed and killed with ether. Pituitary, thyroid and adrenal glands were removed rapidly, blotted to remove surface moisture and weighed to nearest .1 mg on a Roller-Smith Torsion balance.

Results. Administration of reserpine did not significantly affect release of thyroidal-I^{131} resulting from daily injections of TSH (Fig. 1). Average hourly release thyroidal-I^{131} (k′4) during period of TSH injections was .00489 ± .00059 as compared with .00529 ± .00040 during period in which both TSH and reserpine were administered. Injections of tapazole and/or reserpine for 10 days did not significantly affect pituitary or adrenal weight (Table I). Thyroid weight, however, was significantly increased over controls in animals treated with tapazole but concomitant administration of reserpine prevented the increase in thyroid weight which resulted from injections of tapazole. Thyroid weight of reserpine-treated rats did not differ significantly from that of controls.

* Contribution from Dept. of Dairy Husbandry, Mo. Agr. Exp. Sta. Journal Series No. 2093. Approved by the Director.

† Postdoctoral Fellow of NIH. Investigation supported in part by research grants from P.H.S.

‡ We are indebted to Jensen-Salsbery Labs., Kansas City, Mo., for TSH and to Eli Lilly and Co., Indianapolis, Ind. for reserpine and tapazole.

TABLE I. Effect of Reserpine and/or Tapazole upon Gland Weight of Female Rats.

Treatment for 10 days	No. of rats	Avg Body wt (g)	Avg Thyroid (mg/100 g)	Avg Pituitary (mg/100 g)	Avg Paired adrenals (mg/100 g)
Control	16	208.3	3.86 ± .18*	4.56 ± .13	25.2 ± .53
Reserpine (10 μg/100 g/day)	15	218.2	4.22 ± .77	4.46 ± .19	23.5 ± 1.34
Tapazole (4 mg/100 g/day)	14	205.0	7.86 ± .34†	4.81 ± .21	25.6 ± 1.75
Reserpine + tapazole	14	219.9	4.42 ± .21	4.74 ± .19	24.0 ± .76

* Stand. error of mean.

† Significant at .1% level from control.

Discussion. It is generally accepted that goitrogenic compounds depress thyroid function by inhibiting organic-binding of I_2 which, in turn, decreases the circulating thyroxine level with a subsequent increase in TSH secretion and enlargement of the thyroid. It is also well known that goitrogen administration results in an apparent increase in thyroidal-I^{131} release rate since the thyroid is unable to hold recycled I^{131} which is released upon degradation of radioactive hormone secreted by the thyroid gland. Reserpine, on the other hand, has been shown to depress thyroidal-I^{131} release rate(5). This suggests that some factor, other than inhibition of organic-binding, is responsible for the antithyroid activity of the drug. Results of the present experiment are consistent with this interpretation. Tapazole injections resulted in a significant increase in thyroid weight over that of controls, however, concomitant administration of reserpine prevented thyroid weight increase. If the mode of action of reserpine was similar to that of goitrogens, it would be expected that thyroid weight would increase rather than decrease when tapazole and reserpine were injected together or, at least, thyroid enlargement should have been evident in animals treated with reserpine alone. These data indicate a suppression of TSH secretion or inactivation of the hormone so that it is ineffective at the thyroid level. The former suggestion appears the more plausible since thyroidal-I^{131} output resulting from exogenous TSH was not affected by reserpine injections (Fig. 1). If inactivation of TSH had occurred, thyroidal-I^{131} release rate would have been depressed since endogenous TSH secretion was effectively blocked by injections of thyroxine. Recent studies, employing enzyme activity of the thyroid as an index of thyroid function, also indicate a reduced TSH secretion upon administration of reserpine(6).

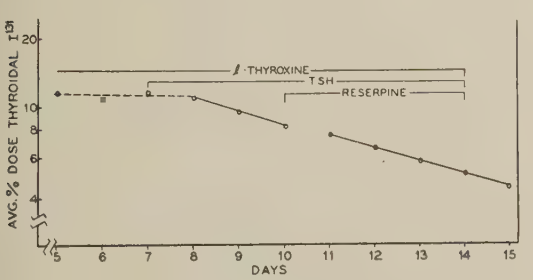

FIG. 1. Effect of exogenous thyroxine, TSH and reserpine upon thyroidal-I^{131} release.

It has also been suggested that reserpine antagonizes thyroxine since the drug prevents increased O_2 consumption which normally results from administration of thyroxine(1,2). Data of the present study are not in accord with this concept since reserpine administration did not increase thyroidal-I^{131} release rate. If the drug antagonizes or inactivates thyroxine, an increase would be expected since the thyroxine block of endogenous TSH secretion would be, at least partially, removed thus resulting in an elevated TSH level and subsequent increased output of thyroidal-I^{131}. It appears, therefore, that the antithyroid activity of reserpine may be attributed to a reduction in TSH secretion *via* the hypothalamo-hypophyseal system since it has been shown reserpine exerts a depressant effect upon neural centers of this area(8,9).

Summary. Reserpine had no significant effect upon thyroidal-I^{131} release rate resulting from injections of thyrotropin (TSH) into young mature female rats whose endogenous secretion of TSH was effectively blocked with thyroxine. In another experiment, reserpine prevented thyroid enlargement which normally results from administration of the goitrogen tapazole. Injections of tapazole and/or reserpine did not significantly affect pituitary or adrenal weight. It appears reserpine

inhibits thyroid function in the rat through suppression of TSH secretion.

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Received July 10, 1959. P.S.E.B.M., 1959, v102.

Effect of Azo-Dye Carcinogenesis on Hexosamine Synthesis in Rat Liver.* (25169)

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The enzyme which catalyzes formation of glucosamine from hexose-6-phosphate and glutamine was first observed in extracts of *Neurospora crassa*(1) and was subsequently demontrated in pig kidney extracts(2), rat liver extracts(3,4) and several tissues of the growing rabbit(5). Recently, hexosamine synthesis was demonstrated in a spectrum of transplantable tumors and it was observed that hexosamine synthesis by transplanted azo dye-induced hepatomas significantly exceeded the synthesis observed with normal liver(6). It was concluded from these observations that either carcinogenesis, subsequent transplantation or both, resulted in increased production of amino sugars by hepatomas when compared to the tissue of origin(6). The purpose of this investigation was to measure the extent of amino sugar synthesis in liver during azo-dye carcinogenesis to ascertain whether the increased amino sugar synthesis was associated with changes occurring during carcinogenesis or transplantation.

Materials and methods. Throughout an experimental period of ca. 150 days, female Holtzman rats were maintained on an *ad lib.* semi-synthetic, riboflavin-deficient diet described by Medes, *et al.*(7). The diet for half the animals contained 0.06% 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) during the first 90 days of the experiment.† Five animals from each group were sacrificed at 30-day intervals during the first 90 days. Then the dye was withdrawn from the diet and dye-fed animals were sacrificed individually when hepatomas were detected by palpation. Five control animals were sacrificed following last determination on dye-fed animals. Livers from both groups were perfused with cold isotonic saline, pooled, homogenized in 0.1 M phosphate buffer(9) pH 6.6, and diluted with buffer to give a tissue nitrogen content of 4 to 5 mg/ml in the reaction mixture. The reaction mixture contained 0.03 M glutamine, 0.02 M glucose-6-phosphate, phosphate buffer (pH 6.6) and homogenate. The conditions just described were optimum for hexosamine synthesis by liver homogenates(6). Aliquots were removed at 0 and 180 minutes and analyzed for total hexosamine content by the Blix(10) modification of the Elson-Morgan method(11). Tissue nitrogen content was determined by Kjeldahl procedure(12).

Results. Glucosamine synthesis appeared the same in livers of both groups throughout the 90-day azo-dye regimen, Table I. Pathological examinations‡ of the precancerous liv-

* The authors express their appreciation to Julia Wheeler and Boyd Howell for technical assistance.

† 3′-Me-DAB was synthesized by the method of Giese, *et al.*(8). The authors are grateful to Merle Maxwell for preparing 3′-Me-DAB.

‡ The authors are indebted to Dr. E. S. Irvine for pathological examinations.

TABLE I. Hexosamine Synthesis by Rat Liver Homogenates during Azo-Dye Carcinogenesis.*

Days	Net hexosamine synthesis† (μg/mg tissue N) Control	Dye-fed
0	32.6	
30	32.6	22.5
60	44.7	21.4
90	28.0	31.5
140	35.3	
120–140 Adjacent liver‡		26.6 ± 3.0
120–140 Primary hepatoma‡		111.6 ±10.9

Reaction mixture: Glutamine 0.03 M, G-6-P 0.02 M, phosphate buffer 0.1 M, pH 6.6. Incubated 3 hr at 38°C.

* 3′-Methyl-4-dimethylaminoazobenzene, fed in a semi-synthetic diet.

† Final total hexosamine concentration minus initial concentration.

‡ Animals sacrificed after hepatomas were detectable by palpation. Values, avg of 3 rats. All other values from livers of 5 animals pooled.

ers indicated absence of neoplastic cells at 60 days on azo-dye and relatively small numbers of neoplastic cells at 90 days. From these data, it was apparent that 3′-Me-DAB exerted no pronounced change upon amino sugar synthesis in precancerous livers. When primary hepatomas and liver adjacent to primary hepatomas were tested for ability to form amino sugars, the results showed primary hepatomas to have activity exceeding either precancerous or control livers by a factor of 3- to 5-fold. The liver adjacent to primary hepatomas had essentially the same activity as control or precancerous livers. Therefore, 3′-Me-DAB carcinogenesis yielded a malignant tissue in which hexosamine formation was significantly elevated compared to its tissue of origin.

Since the magnitude of hexosamine synthesis in the primary hepatomas compared favorably with the synthesis previously observed with transplanted hepatomas(6), it appeared that increased amino sugar synthesis was attributable to biochemical changes induced by 3′-Me-DAB carcinogenesis rather than biochemical changes induced by subsequent transplantation of primary hepatomas. The present observation linking increased amino sugar synthesis with azo-dye carcinogenesis together with: (a) previous observations of mucopolysaccharide biosynthesis by neoplastic tissues(13,14) and (b) demonstration of hexosamine synthesis by transplanted tumors of both epithelial and connective tissue origin (6), supports an hypothesis that amino sugar metabolism constitutes a prominent biochemical feature in neoplastic tissues.

Summary. Rats were fed 3′-methyl-4-dimethylaminoazobenzene in a semi-synthetic diet to determine whether potential for hexosamine synthesis was altered in the precancerous liver and primary hepatoma. Throughout 90 days of dye feeding, no appreciable change was observed in livers of control or dye-fed animals. When primary hepatomas and liver adjacent to primary hepatomas were studied, amino sugar synthesis by primary hepatomas exceeded the synthesis of control or precancerous livers 3- to 5-fold. Activity in liver tissue adjacent to hepatomas was essentially the same as that observed in control or precancerous livers. It was concluded that increased amino sugar synthesis observed in hepatomas compared to liver was associated with biochemical changes occurring during carcinogenesis.

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Received July 13, 1959. P.S.E.B.M., 1959, v102.

Effect of pH on Twitch Facilitating Potency of 3-Hydroxyphenyltriethylammonium Ion.* (25170)

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Administration of certain quaternary ammonium compounds to indirectly stimulated nerve muscle preparations results in a marked increase in response to single supramaximal stimuli. A recent report(1) presented experimental evidence that the facilitation is the result of an action of drugs on the motor nerve terminal. Under the influence of these compounds the nerve responds to a single stimulation shock with a brief repetitive discharge, which, in turn, causes a brief tetanic contraction of the muscle. The agents employed were a series of phenyltrialkylammonium ions. The twitch facilitation and repetitive discharge in the nerve, however, were produced only by compounds bearing a meta hydroxy group. The presynaptic action was particularly pronounced with 3-hydroxyphenyltriethylammonium ion (3 OH PTEA). This compound has a pKa of 8.03 (Gill, E., personal communication). In biologic solutions, therefore, it exists as a mixture of ionized and unionized phenol. The present study was undertaken to determine the relative importance of 2 species in producing the twitch facilitation. This was done by comparing the twitch facilitating potency of 3 OH PTEA on the rat phrenic nerve diaphragm preparation in media of pH 7.0, 7.5, and 8.0. Since the proportion of molecules with an ionized hydroxyl hydrogen increases with increasing pH it was anticipated that changes in the apparent potency with pH would reveal whether the ionized form or the unionized form was the more potent in producing potentiation. To obtain an estimate of amount of change which might occur under these extremes of pH, independent of the presence of 3 OH PTEA, a comparative study was done employing a compound which is not affected by pH, tetraethylammonium ion (TEA). Although the mechanism of action of this compound has not been investigated it is also known to increase twitch height in nerve-diaphragm preparations(2).

Method. Diaphragms obtained from 125 g male rats of the Long-Evans strain were employed in a procedure similar to that described by Bulbring(3). The bathing medium contained, in mmols/L, Na^+, 142; K^+, 5.9; Mg^{++}, 1.2; Ca^{++}, 2.5; Cl^-, 127; HCO^-, 25; SO_4^{--}, 1.2; PO_3^{---}, 1.2 and glucose 200 mg%. It was constantly bubbled with 95% O_2 and 5% CO_2. Under these conditions this solution had a pH of 7.5. Solutions of pH 7 and 8 were obtained by modifying the amount of $NaHCO_3$ used in preparing the medium. Temperature was maintained at 37°C by a water jacket about the chamber. Supramaximal stimuli in the form of square waves of 0.1 msec duration were delivered to the nerve from a Grass Stimulator at the rate of 12/minute. Twitches were recorded on a kymograph by a light isotonic level. After a control period had been recorded the drug, dissolved in 0.1 to 1 ml of distilled water, was rapidly added to the 100 ml of medium contained in the bath. The actions of both agents were fully reversible upon washing the preparation with fresh Krebs' solution. Each diaphragm, therefore, was used to test several concentrations of a drug at each pH level. The sequence in which the combinations of concentrations and pH were tested was determined at random prior to each experiment.

Results. The results are presented in Figs. 1 and 2. The 2 agents, 3 OH TEPA and TEA, had a similar, dual action on the nerve diaphragm preparation. Twitch height was increased in presence of low concentrations of the material, but high concentrations produced a blockade of neuromuscular transmission. Intermediate concentrations produced a biphasic response. Initially the twitch was increased in height, but in time this was superseded by a blockade. In the Figures, maximal

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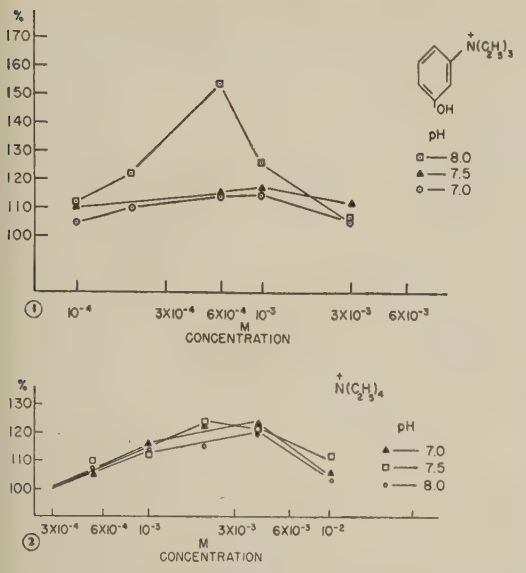

FIG. 1. Effect of pH on concentration response curve for 3-hydroxyphenyltriethylammonium ion. Response is height of twitch of rat phrenic nerve diagram preparation after addition of drug, expressed as percent of control.

FIG. 2. Effect of pH on concentration response curve for tetraethylammonium ion. Response is height of twitch of rat phrenic nerve diaphragm preparation after addition of the drug, expressed as percent of control.

twitch height obtained, expressed as percent of control, is plotted on the ordinate and concentration of drug in the medium is plotted on a logarithmic scale on the abscissa. Each point illustrated represents the mean of 4 to 6 observations.

Over the concentration ranges illustrated both compounds produced an increase in twitch response. At each point the response was significantly greater than control ($p < 0.05$). Concentrations lower than those illustrated produced no significant effect and higher ones rapidly produced a blockade without significant preceding facilitation. At pH 7.0 and 7.5 the increase due to presence of the 3 OH TEPA (Fig. 1) was small and of approximately the same magnitude as that produced by TEA (Fig. 2). At pH 8, however, the potentiation produced by 3 OH TEPA, in the concentration range of 2×10^{-4} to 1×10^{-3}, was significantly greater ($p < 0.05$) than that observed at pH 7.0 and pH 7.5. A similar pH effect did not exist for TEA.

Discussion. It is apparent, therefore, that although presence of a hydroxyl group in a compound is not a pre-requisite to twitch facilitation in this preparation, the ionized form of the phenol is considerably more potent in this regard than is the unionized form. Consequently, the hydroxyl group cannot be contributing to the action by forming a hydrogen bond between the phenol hydrogen and groups in the receptor. Any binding which may occur between the hydroxyl group and the receptor must either be effected by means of electron pairs of oxygen or be an electrostatic attraction between the compound and the receptor. In this regard the binding to presynaptic receptors appears to be quite different from that described by Wilson and Quan (4) for the reaction between close analogs of 3 OH PTEA and the acetylcholinesterase enzyme. On the basis of studies of the pH dependence of the reaction of 3-hydroxyphenylethyldimethylammonium(5) and 3-hydroxyphenyltrimethylammonium(4) ions with the enzyme they concluded that the unionized phenolic hydroxy group contributed more to the binding than the ionized form did and that this probably occurred through the formation of a hydrogen bond.

Summary. Ability of 3-hydroxyphenyltriethylammonium ion to facilitate the response of rat phrenic nerve diaphragm to single supramaximal stimuli has been tested for pH dependence. It is concluded that the phenolate ion is more active in this regard than is the unionized phenol, and that, therefore, the receptor sites for this action on the motor nerve terminals differ from those ascribed to the cholinesterase enzyme.

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Received July 13, 1959. P.S.E.B.M., 1959, v102.

Hemorrhagic Reactions in Animals Sensitized with Ovalbumin in Adjuvants Containing Mycobacteria II. Relation to "Delayed" Hypersensitivity. (25171)

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It has been shown that in guinea pigs sensitized by injection of egg albumin incorporated in paraffin oil (incomplete adjuvant), subcutaneous injection of a very large amount (50 mg) of egg albumin results in protracted anaphylactic shock(1,2) and there is scant if any skin reaction at site of injection of antigen(3). The absence of Arthus reactions may be due to certain components of the shock syndrome, for example, fall in blood pressure(3). When similar experiments are made employing water-in-oil emulsions containing *mycobacteria* (complete adjuvants) in sensitizing the guinea pigs, both the systemic and local responses to an eliciting injection of egg albumin are different. During the very protracted systemic reaction, which may show a combination of "delayed" (DTH) and "immediate" (ITH) reactions, there is a massive hemorrhage at site of injection of egg albumin beginning in the subcutaneous tissue and spreading to the skin(4). The interaction of DTH and ITH systemic reactions is manifested by a combination of the thermal responses characteristic of these reactions. In this connection, it is of interest that Uhr and Brandriss have shown that anaphylactic shock (lowering of body temperature) counteracts the systemic DTH reaction (elevation of body temperature) in guinea pigs previously sensitized by the intracutaneous injection of very small amounts of antigen (combined with excess antibody in specific precipitates)(5). The association of the massive hemorrhagic reaction with the presence of mycobacteria in the adjuvant emulsions suggests that the hemorrhagic reaction is related to DTH(6). The following experiments were designed to shed light on the mechanism of the massive hemorrhagic reaction.

Materials and methods. Adult male or female Hartley guinea pigs were sensitized by a single dose of 2.5 mg of egg albumin (Ea). The antigen incorporated in paraffin oil adjuvants with or without mycobacteria was injected by intracutaneous, subcutaneous, intramuscular or intraperitoneal routes (sensitization schedules are included in tables). For details on the preparation of adjuvant mixtures see(6). Some of the guinea pigs were immunized with precautions taken to avoid contaminating the skin with adjuvant emulsions. For this purpose, a 15 gauge short needle was pushed through the skin in anesthetized guinea pigs and injections were made through a long (3½ inch) 18 gauge needle with regular (I.M.) or cut-off (S.C. or I.P.) end introduced through the 15 gauge needle. The long needle was wiped by pinching the subcutaneous tissue on withdrawal after S.C. injections. Serum antibody titers were determined by the passive cutaneous anaphylaxis (PCA) technic. The ear vein of the guinea pigs was split with a scalpel. Blood was taken by and diluted in a white blood cell pipette. Two or more guinea pigs were used to test each sample of plasma (2-fold dilutions; 0.1 ml of dilution injected intracutaneously) and 8-10 mm of blueing was taken as the threshold reaction. Guinea pigs were injected with the eliciting dose of 50 mg of Ea subcutaneously on the flank 4 to 6 weeks after the sensitizing injections. The temperatures of guinea pigs were taken before the eliciting injection of antigen and during the systemic reaction that followed the injection. For this purpose a thermistor thermometer (TRI-R Instruments, Long Island City, New York) was used.

Results. Local hemorrhagic reaction. It has been found necessary to incorporate the antigen in the paraffin oil emulsion containing mycobacteria in order to induce DTH to that antigen. If the antigen is injected at one site in water-in-paraffin-oil emulsion and *M. tuberculosis* in water-in-paraffin-oil emulsion is injected at a remote site the mycobacteria

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TABLE I. Effect of Injection of Mycobacteria and Egg Albumin at Separate Intracutaneous Sites during Sensitizing Procedure.

G.P. No.	Sensitization at separate sites: Egg albumin in oil (Nuchal site)	Sensitization at separate sites: *M. tuberculosis* in oil (Nuchal site)	Skin hemorrhage, mm × mm	PCA antibody titer (recip.)
1	Right*	Left*	0	6400
2			0	6400
3			0	3200
4			0	3200
5			0	ND
6			0	ND
7	Right and left	Right and left	40 × 30	12800
8			90 × 50	25600
9			80 × 60	"
10			50 × 50	"
11			70 × 50	6400
12			65 × 35	12800
13			0	3200
14			0	12800

* Injections made intracut. right or left of midline running parallel to spinal column.

Plan of Injection Sites

G.P. No. 1-6		G.P. No. 7-14	
Mycobact. 0.5 mg	Ea 1.25 mg	Ea 1.25 mg	Mycobact. 0.5 mg
Mycobact. 0.5 mg	Ea 1.25 mg	Mycobact. 0.5 mg	Ea 1.25 mg

do not alter the sensitization (*i.e.* do not induce DTH to the antigen)(7). Tables I and II show the data on reactions at the site of the subcutaneous injection of 50 mg of Ea in guinea pigs which had been sensitized with Ea in oil emulsion at one site and with *M. tuberculosis* in oil at another. These animals did not react with gross hemorrhages of the type seen in Fig. 1 unless both sensitizing injections were on the same side of the nuchal midline. When *M. tuberculosis* in oil and Ea in oil were injected (at separate sites) on the same side of the midline, antibody titers were somewhat higher than in animals injected with Ea on the side of the midline opposite from the *M. tuberculosis*. However, when guinea pig anti-Ea antibody was injected intravenously for passive sensitization of guinea pigs sensitized only to *M. tuberculosis* and into some animals actively sensitized to both Ea and tubercle bacilli to increase the titer of antibody, neither hemorrhagic nor inflammatory reactions were elicited when 50 mg of egg albumin were injected subcutaneously.

Injection of antigen by the intracutaneous route has been found particularly effective for inducing DTH(8,9,10). Table III shows the effect of route and site of immunization on the hemorrhagic skin reactions. Intracutaneous injection of Ea in complete adjuvant was effective in sensitization of guinea pigs whether

TABLE II. Effect of Injection of Mycobacteria at One Site and Egg Albumin at a Remote Site during Sensitizing Procedure.

G.P. No.	Sensitization at separate sites: Egg albumin in oil (Route*)	Sensitization at separate sites: *M. tuberculosis* in oil (Route*)	Skin hemorrhage, mm × mm	Passive antibody† (PCA units)	Antibody titer at time of eliciting inj. (recip.)
1	I.C.	S.C.	15 × 15‡	0	12800
2			0	0	3200
3			10 × 10‡	500,000	6400
4			0	"	12800
5	(None)		0	"	ND§
6	(")		0	"	ND§
7	I.P.	I.C.	0	0	1600
8			0	0	"
9			0	0	"
10			0	0	"
11			0	0	3200
12			0	0	"
13			0	0	"
14			0	0	"

* I.C. = intracut. inj. in nuchal area; S.C. = subcut. inj. on flank; I.P. = intraper. inj.

† Inj. intrav. just before bleeding from the ear. The eliciting inj. of 50 mg Ea followed taking of blood. A PCA unit causes blueing 8-10 mm in diameter.

‡ Resembled partially inhibited Arthus reaction (see 3). Avg size of hemorrhagic reaction is 2800 mm² in fully sensitized guinea pigs (see (4) and Table IV).

§ Phenergan (2 mg) inj. I.P. 1 hr before eliciting inj. to prevent possibility of acute anaphylactic shock (see 2). Antibody titer in circulation not determined at time of challenge.

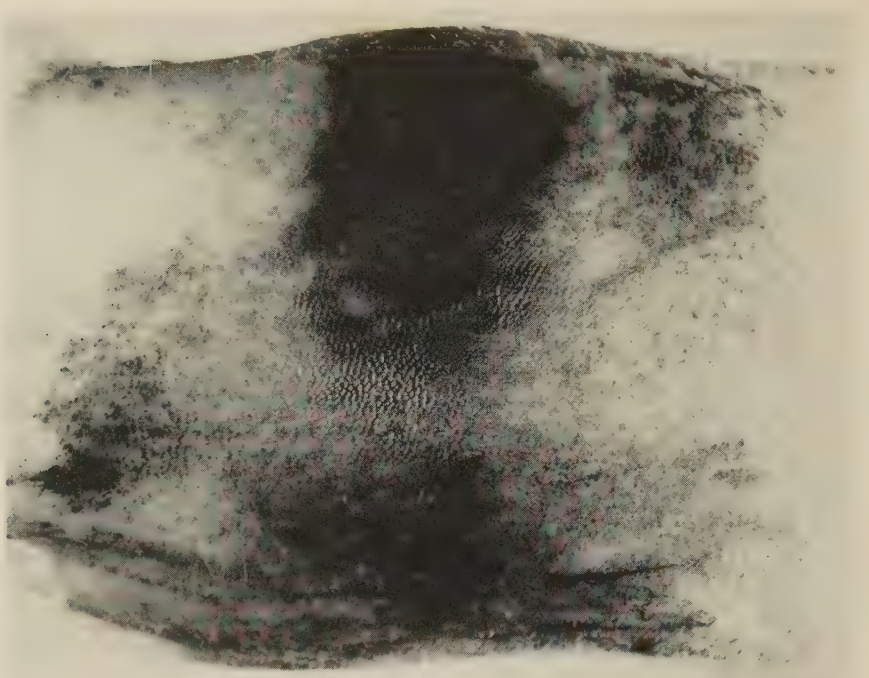

FIG. 1. Hemorrhagic reaction 24 hr after subcutaneous injection of 50 mg of egg albumin in a guinea pig sensitized with egg albumin in complete paraffin oil adjuvants containing mycobacteria.

the site chosen for intracutaneous injection was the flank (5 of 5 guinea pigs showed hemorrhages) or the nuchal area (8 of 9 guinea pigs showed hemorrhages).

TABLE III. Effect of the Site and Route of Sensitization on the Incidence of Hemorrhagic Skin Reaction.*

Route	Area	No. of G.P. Hem. skin reaction	PCA antibody titer (recip.) Mode or median	Max
S.C.	Flank	2/12	ND	ND
"	"	0/10†	3200	12800
I.C.	"	5/5	6400	"
S.C.	Nuchal	7/8 ‡	12800	"
I.C.	"	8/9	‖	‖
I.M.	"	3/5 §	12800	12800
"	Thigh	6/6 †	6400	"
I.P.		2/5	12800	25600

* All guinea pigs inj. subcut. with 50 mg Ea as eliciting dose. Sensitizing dose contained 2.5 mg Ea and 1 mg *M. tuberculosis*.

† Inj. with precautions to avoid contamination of skin with emulsion.

‡ One guinea pig died at 4½ hr. At the time, no hemorrhage in skin, but massive hemorrhage in peritoneal wall.

§ Three guinea pigs were found dead 20 hr after eliciting inj. One of these showed hemorrhagic skin reaction, the other 2, no hemorrhage in skin but massive hemorrhage in subcut. tissue and peritoneal wall.

‖ Titers in guinea pigs similarly sensitized: Mode, 12800; maximum, 25600.

Sensitization by the subcutaneous route varied with regions of injection. After sensitization with Ea combined with complete adjuvants injected subcutaneously on the flank, a subcutaneous injection of 50 mg of Ea did not elicit a massive hemorrhage in the skin, but hemorrhages were observed in the subcutaneous tissue and in the outer part of the peritoneal wall. The hemorrhagic skin reactions seen in a small percentage of guinea pigs sensitized by the subcutaneous route on the flank were not observed in animals sensitized by subcutaneous injections made with precautions to avoid contamination of the skin with emulsion. However, subcutaneous injection of antigen in the nuchal area sensitized guinea pigs so that reactions were observed in the skin as well as in the subcutaneous tissues after a subcutaneous eliciting injection.

Hemorrhagic skin reactions were elicited in most guinea pigs sensitized by the intramuscular route (thigh or nuchal area), and less frequently (2 of 5) in guinea pigs sensitized by the intraperitoneal route.

TABLE IV. Quantity of *Mycobacterium tuberculosis* and Hemorrhagic Reaction.

	Male guinea pigs			Female guinea pigs		
M. tuberculosis, mg	No. with hem. skin reaction	Size of hemorrhagic reaction in mm² Avg*	Max	No. with hem. skin reaction	Size of hemorrhagic reaction in mm² Avg	Max
.1	3/4	1800	3000	ND†	—	—
.075	3/5	"	3500	7/7	2800	4500
.04	ND	—	—	5/8	1500	3500
.03	4/5	1200	2100	3/5	1850	4500
.02	0/4	(0)	(0)	4/5	1200	3150

* Avg size of hemorrhagic area determined counting only those guinea pigs with hemorrhages in the skin.
† Not done.

It is of interest to compare the amount of killed and dried mycobacteria necessary to induce aspermatogenesis or allergic encephalomyelitis with the amount of mycobacteria required for the induction of massive hemorrhagic reactions. Table IV shows that 0.03 mg of mycobacteria in the adjuvant emulsion was effective in sensitization of male guinea pigs for eliciting hemorrhagic reactions. Females were apparently more susceptible to sensitization of this type. With 0.075 mg of mycobacteria in the sensitizing emulsion, female guinea pigs were sensitized so that maximum size reactions were elicited; in males which received this dose, reactions were not maximal. The threshold dose in males corresponded well with the 0.02 to 0.04 mg of *M. tuberculosis* necessary for inducing aspermatogenesis or allergic encephalomyelitis (only males were used in those experiments(11)).

Desensitization or decrease in reactivity. A subcutaneous injection of 50 mg of Ea 24-48 hours after eliciting of a hemorrhagic reaction in sensitive guinea pigs failed to elicit another massive hemorrhage (in animals sensitized with near threshold doses of tubercle bacilli) or produced a less severe reaction (usually in animals sensitized with relatively large amounts of mycobacteria). PCA tests of the serum of sensitized guinea pigs injected 24 hours previously with 50 mg of Ea showed the presence of at least 10% of the original antibody activity.

Tuberculin sensitivity. In guinea pigs sensitized by the intracutaneous route with Ea in complete adjuvants containing *M. tuberculosis*, subcutaneous or intracutaneous injection of relatively large amounts of purified protein derivative (PPD) (0.1 to 0.25 mg) elicited strong tuberculin reactions, but did not cause hemorrhagic responses.

Systemic reactions. The shock seen in guinea pigs sensitized with Ea in complete adjuvants by the intracutaneous route is very prolonged. However, its course with regard to rectal temperature differed somewhat from the very protracted shock observed in animals sensitized by adjuvant emulsions not containing mycobacteria. In the former group (complete adjuvants) body temperatures were normal or showed a slight rise over a 24-hour period; in the latter group (incomplete adjuvants) temperatures were normal or subnormal.

Histological observations. (Figs. 2, 3, 4, 5). In guinea pigs sensitized with Ea combined with *complete* adjuvants and challenged with subcutaneous injection of 50 mg Ea, at 24-48 hours hemorrhage and edema were conspicuous in the skin and subcutaneous tissue. The collagen and muscle fibers were swollen and in part disrupted. Inflammatory cells were conspicuous by their absence. In guinea pigs similarly treated but sensitized with Ea combined with *incomplete* adjuvants, there was edema but no hemorrhage. In addition, there was very slight infiltration of the skin and subcutaneous tissue with thrombotic blood vessels in the skin.

Discussion. These experiments show that the hemorrhagic reaction previously reported (4), which was elicited by injection of a large amount of antigen in guinea pigs sensitized using adjuvant emulsions containing mycobacteria (complete adjuvants) did not occur when the sensitizing antigen was injected

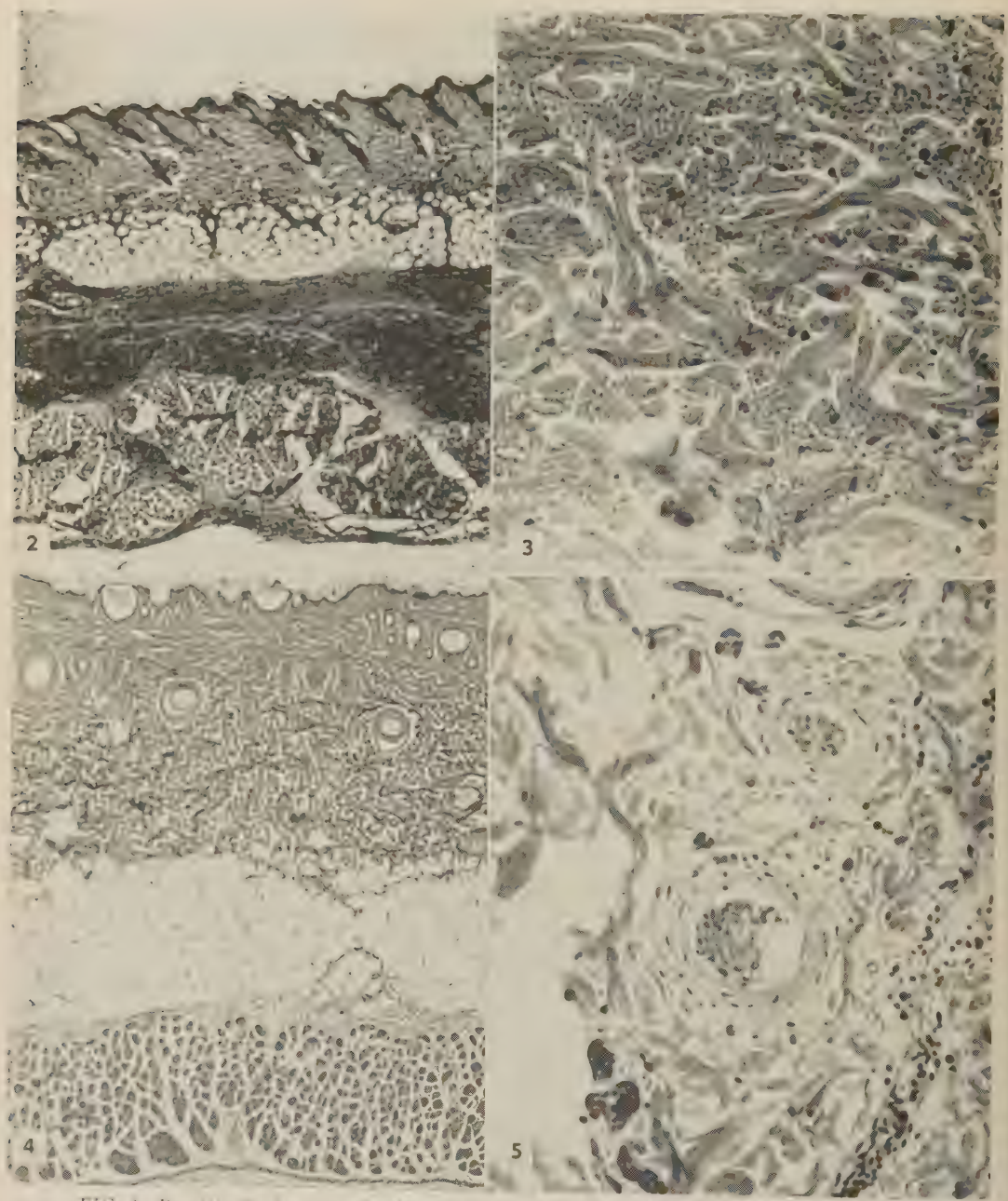

FIG. 2. Reaction in the skin of guinea pig sensitized to Ea using *complete adjuvants*. Hemorrhage and edema in skin and subcutaneous tissue. Giemsa 235 ×. Dark staining masses are RBC.

FIG. 3. Reaction in the skin and subcutaneous tissue of guinea pig sensitized to Ea using *complete adjuvants*. Hemorrhage and edema in subcutaneous tissue. Hematoxylin and eosin 235 ×.

FIG. 4. Reaction in the skin and subcutaneous tissue of guinea pig sensitized to Ea using *incomplete adjuvants*. Edema and very slight inflammatory infiltrate in skin. Hematoxylin and eosin 235 ×.

FIG. 5. Reaction in the skin of guinea pig sensitized to Ea using *incomplete adjuvants*. Thrombotic blood vessels in skin. Edema, slight and very slight infiltration. Hematoxylin and eosin 235 ×.

at one site and the mycobacteria at another site across the nuchal midline. Since DTH (*i.e.* induction of aspermatogenesis) does not occur when tissue antigens in water-in-oil

emulsion are injected at one site and mycobacteria in oil are injected at a remote site (7) there appear to be similarities between the pathogenesis of auto-allergic diseases and the hemorrhagic reaction. This observation also rules out the possibility that the hemorrhage in guinea pigs is due to increased vulnerability of blood vessels caused by the presence of mycobacterial components and suggests that the effect of killed tubercle bacilli is due to their modification of the process of sensitization to Ea. Since hemorrhagic reactions were observed in animals with mycobacteria and Ea injected at separate sites on the same side of the nuchal area, this modification may occur, at least under certain conditions, in the draining lymph nodes.

There is evidence that serum antibody is not the decisive factor in eliciting the hemorrhagic reaction. Individual animals sensitized with the Ea emulsified in oil omitting mycobacteria had titers of circulating antibody equal to those of individual guinea pigs sensitized using complete adjuvants, yet they did not react with hemorrhages. Furthermore, antibody administered passively to guinea pigs previously injected only with *M. tuberculosis* in oil did not cause sensitivity of the type described above.

Since the hemorrhagic reaction appears to be related to DTH it is of interest to determine the quantity of mycobacteria needed to sensitize for such a response. The amount, *i.e.*, 0.03 mg, is of the same order as the threshold dose for inducing aspermatogenesis and allergic encephalomyelitis (11) which are generally considered to be pathological manifestations of DTH to tissue antigens.

Guinea pigs sensitized by *subcutaneous* injection of Ea incorporated in *incomplete* adjuvants usually died during protracted anaphylactic shock elicited by subcutaneous injection of large amounts of Ea (1,2). This protracted shock is characterized by hypothermia. Guinea pigs sensitized by *intracutaneous* injection of Ea incorporated in *complete* adjuvants did not die, and showed normal temperatures or a rise in temperature. Reversal of the hypothermia of anaphylactic shock, probably caused by a systemic DTH reaction in the guinea pigs sensitized using complete adjuvants, does not explain the survival of these animals since guinea pigs sensitized by the *intracutaneous* route using *incomplete* adjuvants also survived. The causes of death after protracted anaphylactic and systemic DTH reactions, and the effects of interaction between them are still unclear, and further investigations in these areas appear necessary before further discussion of the problems involved.

In this connection the experiments of Uhr and Brandriss (5) are of interest. Guinea pigs were sensitized to diphtheria toxoid first by injection of trace amounts of toxoid combined with excess antitoxin leading to DTH (12). These animals responded to injection of toxoid by increases in body temperature. Subsequently guinea pigs which had been similarly sensitized were given guinea pig serum containing antitoxin or were stimulated to form circulating antitoxin. When these animals were tested for systemic reactions to toxoid, they responded with a drop of body temperature. Apparently the anaphylactic (ITH) response was predominant over the previously established DTH. In contrast, our experiments demonstrate a predominant DTH thermal response, perhaps reflecting a higher degree of DTH in animals sensitized using Ea incorporated in complete adjuvants (1.0 mg *M. tuberculosis*).

Summary and conclusions. 1. Massive hemorrhages in skin and subcutaneous tissue of guinea pigs sensitized with egg albumin incorporated in water-in-oil adjuvants containing *mycobacteria* caused by subcutaneous injection of large amounts of egg albumin, failed to occur in guinea pigs which had been sensitized by injection of egg albumin at one site and mycobacteria in oil at a remote site. This indicates that presence of killed tubercle bacilli at site of injection of antigen modifies the process of sensitization to ovalbumin, manifested by hemorrhage when large amounts of egg albumin were injected subcutaneously. 2. Guinea pigs sensitized by injection of egg albumin incorporated in complete adjuvants *on the flank* had hemorrhagic *skin* reactions if the sensitizing injection was intracutaneous, but not if it was made subcutaneously. Injections in the nuchal area were

effective whether intracutaneous, subcutaneous or intramuscular. 3. For inducing hemorrhagic skin reactions, 0.03 mg of killed and dried *M. tuberculosis* was necessary in the adjuvant emulsion used for sensitization of male guinea pigs. For male guinea pigs, 0.02 mg of mycobacteria was not effective, but was sufficient for female animals. 4. In guinea pigs sensitized by intracutaneous route using 2.5 mg of egg albumin and 1 mg *M. tuberculosis*, the systemic reaction elicited by subcutaneous injection of 50 mg of egg albumin was not characteristic of protracted anaphylaxis. Body temperatures were normal or slightly above normal, indicating that anaphylactic shock hypothermia is "neutralized" by concurrent systemic "delayed" reactions.

The authors express thanks to Dr. E. M. Lerner for cooperation in preparation of microphotographs.

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Received June 12, 1959. P.S.E.B.M., 1959, v102.

A Fibrinogen Precipitating Factor (FPF) of Group A Streptococci. (25172)

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During experiments designed to test antigenicity of a preparation of Group A, type 1 streptococcal M protein, plasma samples from immunized rabbits were set up in capillary precipitin tubes against homologous M protein. A massive precipitate appeared in each tube 5 minutes after preparation. The magnitude of these reactions far exceeded reasonable expectation of the amount of type-specific antibody present. Furthermore, normal plasma reacted in the same manner, and sera from normal and immunized rabbits proved non-reactive. The nature of this reaction is herein examined.

Materials and methods. Bacterial strains and extracts. Streptococcal, pneumococcal and staphylococcal cultures were obtained from human sources in the course of other studies. All streptococcal cultures were typed by capillary precipitin technic of Swift, Wilson and Lancefield(1), then stored in the lyophilized state. Cultures were initiated in modified Todd-Hewitt broth† and plated for purity. Crude acid extracts were prepared by heating suspensions of bacterial cells in boiling water bath at pH 2(2). M extracts were prepared by repeated alcohol precipitation of crude extracts(3). A partially purified preparation of M protein was prepared by the method of Lancefield and Perlmann(4) from Type 1 Streptococci, strain #2788, kindly supplied, as phenolized cells, by Dr. C. P. Hegarty of Merck, Sharp, and Dohme Labs. Electrophoretic examinations of this material revealed migration as a single peak, but with sufficient boundary spreading to suggest inhomogeneity. Streptococcal T extracts were prepared by slight modification of the method of Pakula(5). Crystalline trypsin in final concentration of 0.01 mg/ml at pH 7.6 was substituted for pancreatic extract. *Enzymes.* Pepsin (Difco 1:10,000) was employed in final concentration of 1% at pH 2. Certain strains and extracts were treated with crystalline trypsin (General Biochemicals, Inc.) to

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† Lean horse meat was substituted for beef hearts.

final concentration of 0.01 mg/ml at pH 7.4. Human fibrinogen (lot #17), obtained from E. R. Squibb and Sons, through the courtesy of American Red Cross, was further purified by the method of Laki(6); the purified material contained 93% clottable protein, and was used in 0.5% concentration in 0.3 M KCl buffer at pH 7.2. *Normal rabbit plasma.* Coagulation of each 5 ml aliquot of blood was prevented with one of the following: 0.05 ml heparin (Abbott 1:1000), 0.5 ml sodium citrate (3.8% solution), 0.5 ml potassium ammonium oxalate solution (2%). Plasma was separated by centrifugation at 1500 g for 15 minutes. *Streptococcal typing sera* were obtained from the Diagnostic Reagents Section, Communicable Disease Center, USPHS, Chamblee, Ga.

Results. Effect of various anticoagulants, and plasmas of different species. In the original observation, partially purified type 1 M protein precipitated with normal rabbit plasma obtained from heparinized blood. Subsequently, oxalated and citrated rabbit plasmas precipitated equally well. Furthermore, all mammalian plasmas tested, *i.e.* man, monkey, guinea pig, rat, and mouse plasma, precipitated strongly with type 1 M protein. Rat plasma behaved differently than the others; an immediate precipitate occurred but disappeared within a few minutes. No activity of M protein against chicken plasma has been found, and addition thereto of varying amounts of normal rabbit serum did not render chicken plasma precipitable. Addition of type 1 M protein to purified human and bovine fibrinogens resulted in immediate, massive precipitates. The material precipitating with plasma or fibrinogen has been designated, for convenience, fibrinogen precipitating factor, or FPF.

The *FPF positive reaction* in capillary tubes appears within 5 minutes at room temperature when purified M protein and fibrinogen are used. This reaction is not inhibited by diisopropylfluorophosphate (DFP), nor will a known positive M protein hydrolyze synthetic substrate, *i.e.,* tosyl-arginyl-methyl ester (TAME).‡ Strongly positive FPF reactions appear as gross floccules throughout the tube; in weaker reactions a fine haze appears. Fibrin-like retraction may be seen with a hand lens after a short time. The precipitated material, examined under phase microscope, consists of considerable amorphous material together with some characteristic fibrin needles.

Occurrence of FPF in various organisms. Subsequent to finding FPF in a purified M protein preparation, it was demonstrated in crude acid extracts of some but not all beta hemolytic streptococci prepared for routine grouping and typing. To determine distribution of FPF among streptococci, pneumococci, and staphylococci, crude acid extracts of 18 hour Todd-Hewitt broth cultures of these organisms were prepared, and tested in capillary tubes against purified human fibrinogen. The tubes were incubated 2 hours at 37°C, then overnight at room temperature. Estimates of amount of precipitates varied from 1+ to 4+. Of 146 strains of beta hemolytic streptococci tested, 131 were of Group A; the remainder were distributed among Groups B, C, D, F, G, and K (Table I). It should be noted that typing of Group A organisms was performed prior to lyophilization and storage; not all strains were typable when the lyophile tubes were opened and cultured. Reactivity of a particular extract with fibrinogen did not correlate, however, with its ability to react with type-specific antiserum. FPF was found in at least one strain of each type of Group A streptococci when 2 or more strains were available for testing, except for 5 typable strains of Type 18; crude acid extracts of these 5 strains proved nonreactive. However, M extracts prepared from 2 of these crude extracts by repeated alcohol precipitation both precipitated (weakly) with fibrinogen.

Of 15 non-group A streptococcal extracts, only one from a Group G organism proved reactive. Acid extracts of 5 coagulase-positive strains of staphylococci, 4 typed strains of pneumococci, and 5 strains of alpha-hemolytic streptococci did not react with fibrinogen or plasma. Eleven T extracts prepared by Pakula's method from FPF positive strains of

‡ These determinations were kindly performed by Dr. Jules P. Gladner, Laboratory of Physical Biology, Nat. Inst. of Arthritis and Metab. Dis., N.I.H., Bethesda, Md.

TABLE I. Distribution of Fibrinogen Precipitating Factor in Extracts of Hemolytic Streptococci, Pneumococci, and Staphylococci.

Organism	Putative type*	No. strains tested	No. of extracts reactive with: Homol. antiserum	Fibrinogen
Hemolytic streptococci				
Group A	1	4	4	3
	2	4	0	3
	3	3	3	2
	4	29	29	28
	5	4	4	2
	6	5	5	3
	8	3	No serum	3
	11	5	5	4
	12	28	28	6
	13	4	0	3
	14	1	1	0
	15	1	1	0
	17	2	0	1
	18	5	5	0
	19	4	3	2
	22	3	1	2
	24	1	1	0
	23	4	3	2
	25	3	0	2
	26	1	1	0
	28	2	2	2
	29	2	2	1
	30	1	1	0
	31	2	2	1
	32	1	1	0
	36	1	1	0
	37	1	1	0
	39	1	1	0
	40	1	1	0
	43	1	1	0
	44	2	No serum	0
	46	1	1	0
	47	1	1	0
Group B		2	2	0
C		4	4	0
D		2	2	0
F		2	2	0
G		4	4	1
K		1	1	0
Viridans streptococci		5		0
Staphylococci		5	All coag. pos.	0
Pneumococci		4	4	0

* All strains typed prior to storage.

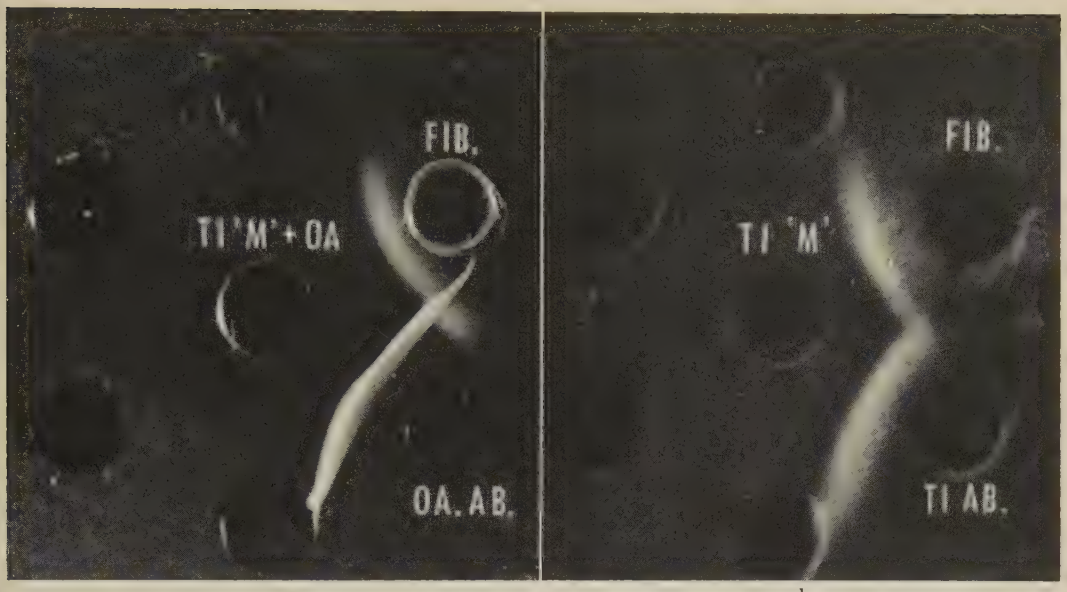
Group A streptococci did not precipitate fibrinogen.

Effect of heat, pH, and proteolytic enzymes. Treatment of partially purified type 1 M protein with pepsin at pH 2 for 2 hours at 37°C with subsequent neutralization, removed both FPF and the type-specific material. Trypsin was similarly effective. FPF could not be acid-extracted from trypsinized streptococcal cells known to contain this factor prior to trypsin treatment. Heating for 35 minutes at 95°C at pH 2 did not affect either FPF or M protein.

Identity. Fibrinogen precipitating factor could not be separated from M protein. Absorption of a 0.1% solution of partially purified Type 1 M protein with an equal volume of 0.5% solution of human fibrinogen completely removed both M protein and FPF; 2 absorptions of M protein solution with equal volumes of type-specfiic antiserum also removed both M protein and FPF. Finally, agar diffusion experiments illustrated in Figs. 1a and 1b demonstrate that FPF and M protein represent 2 activities resident in the same moiety. In Fig. 1a, a mixture of 0.1% M protein in saline, and 0.5% saline solution of ovalbumin, in ratio of 4:1, was pipetted into the center well. A solution of 0.5% fibrinogen was placed in the well marked "FIB.," and undiluted rabbit antiovalbumin serum was placed in the well marked "OA. AB." The reaction between FPF and fibrinogen in no way interfered with the immune precipitate formed between ovalbumin and homologous antiserum. However, when M protein alone is pipetted into the center well, (Fig. 1b), type 1 antiserum placed in the well marked "Tl. AB.", and fibrinogen placed in the well marked "FIB.," the reaction between FPF and fibrinogen interferes with the immune precipitate line, which ends abruptly at the intersection of the 2 precipitates. It is clear that fibrinogen is capable of absorbing M protein so that none is available for reaction with type-specific antiserum in the region peripheral to the FPF-fibrinogen precipitate line.

Inhibition of FPF reaction. The weak reactivity of M extracts of two Type 18 streptococcal strains, and the lack of reactivity of crude acid extracts of these same strains suggested the presence of inhibitors. Since both these strains were highly mucoid, possible inhibition by hyaluronic acid was investigated as follows: a 3 ml suspension of known FPF positive Type 1 bacterial cells was divided into 3 equal samples. To Sample I, 1 ml of 0.1% hyaluronic acid§ was added; to Sample

§ Highly purified potassium hyaluronate from human umbilical cord (Schering Corp., West Berlin) was kindly supplied by Dr. Emily Emmart, Lab. of Pharmacol. and Toxicol., Nat. Inst. of Arthritis and Metab. Dis., N.I.H.



a. b.

FIG. 1. Agar-gel precipitin reactions of type 1 M protein with fibrinogen and type-specific antiserum. T1 M, .1% Type 1 M protein; FIB., .5% Fibrinogen; OA., .5% Ovalbumin; T1 AB., Type 1 Antiserum; OA. AB., Ovalbumin Antiserum.

II, 1 ml of saline was added; the third sample was untreated. Reaction of each sample was lowered to pH 2 with N HCl and they were extracted in a boiling water bath for 10 minutes. Subsequent to cooling, Sample III received 1 ml of hyaluronic acid. All samples were neutralized with N/5 NaOH to pH 7.4 and volumes equalized with saline. These samples were set up in capillary tubes with fibrinogen and with type-specific antiserum. All extracts reacted strongly with type-specific antiserum. Sample I reacted minimally with fibrinogen when compared with the other 2 samples. The presence of hyaluronic acid prior to acid extraction at 95°C impaired reactivity of the resulting extract with fibrinogen. Addition of hyaluronic acid to Sample III after acid extraction had no apparent effect.

Discussion. An active principle has been found in various streptococci which precipitates the plasmas of several different animals. This material is closely related, if not identical to M protein. Reactivity of crude streptococcal extracts with type-specific antiserum did not correlate, however, with ability to precipitate fibrinogen. An explanation for this lack of correlation may be the interference, by inhibitors, with the demonstration of FPF in typable extracts. The experimental data favor this explanation: 1) Purification of two type 18, FPF negative, crude extracts by repeated alcohol precipitation resulted in fibrinogen reactive substances. 2) Addition of hyaluronic acid to a known FPF positive suspension of bacteria prior to acid extraction resulted in virtual absence of FPF activity in the crude extract. Since strains of streptococci vary considerably in production of hyaluronic acid, this material may be one explanation for the presence of type-specific substance in an extract devoid of fibrinogen activity.

Reaction of streptococcal M protein with fibrinogen suggests an analogy with staphylococcal coagulase. Although both materials are present chiefly in virulent strains, inhibit phagocytosis(7,8), are trypsin sensitive, and poorly antigenic(9,5), they are not analogous. M protein is located in the cell wall, while staphylocoagulase is an extracellular product. Coagulase activity results in a typical fibrin gel, whereas FPF precipitates with fibrinogen. Furthermore, coagulase requires a serum co-factor, coagulase reacting factor (CRF), for fibrinogen conversion; this

factor is not present (in adequate amounts) in rat, mouse, and chicken plasma(10). Our experience, cited above, shows that FPF precipitates rat and mouse plasma. Chicken plasma reacts with neither coagulase nor FPF; however, addition of normal rabbit serum renders chicken plasma clottable with coagulase, but does not impart FPF reactivity.

The significance of the fibrinogen precipitating factor in streptococcal infections and the non-suppurative sequalae of such infections is the subject of current investigations.

Summary. 1) An active factor was found in streptococci which precipitates various mammalian plasmas, and human and bovine fibrinogens. Fibrinogen precipitating factor (FPF) was found in crude acid extracts of several strains of group A, and one strain of group G streptococci; extracts of staphylococci, pneumococci, and viridans streptococci have proven non-reactive. 2) Streptococcal M protein and FPF appear inseparable, although presence of hyaluronic acid in a suspension of streptococcal cells prior to acid extraction may inhibit the fibrinogen precipitating reactivity of the extract, without significantly affecting the typing reaction. FPF and staphylocoagulase share certain properties, but they are not analogous.

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Received July 9, 1959. P.S.E.B.M., 1959, v102.

Properdin Levels in Splenectomized Persons. (25173)

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Our previous studies(1) showed that antibody response to tularemia vaccine in 92 of 105 splenectomized persons was similar to that observed in 47 normal controls. Failure of 13 persons to exhibit antibodies was attributed to the underlying disease for which splenectomy was performed rather than to absence of the spleen. This present study is concerned with determination of properdin levels in the same 105 splenectomized subjects.

Materials and methods. The 105 splenectomized persons included 35 males and 70 females, 2 to 77 years old and 2 months to 18 years post-splenectomy. Blood samples for properdin determinations were obtained prior to tularemia vaccination(1). Healthy non-splenectomized controls consisted of 100 males and 13 females, 18 to 40 years of age. All blood samples were centrifuged 5-7 minutes at 2000-2200 rpm in an International, Size 2 centrifuge and the serum frozen and stored at −22°C until tested. Properdin levels were determined by the Pillemer zymosan assay technic(2). Serum reagents RP and R3 were prepared from pools of normal human serum(3) and standardized by the Pillemer method(2). Serial 2-fold dilutions of test sera(4) were used. The amounts of zymosan* found optimal were 1 mg and 2.5 mg/ml of normal human serum in preparation of R3 and RP respectively, and 6 mg/ml of RP in the assay proper. Sensitivity and validity of the test were controlled by daily assays of 1 serum sample of known properdin content and 2 synthetic samples prepared by adding known

* Nutritional Biochemicals Corp., Cleveland, O.

amounts of properdin† to RP. The C'3 content of RP was always between 90 and 150 units/ml and degree of hemolysis in control tubes was always within permitted limits. Suitability of freshly prepared reagents was established by re-assaying 20 samples of known properdin content. The degree of surveillance required by this test is represented by the fact that our report contains results of 328 assays, whereas almost 200 additional assays were performed for control purposes only. The nature of our study necessitated certain departures from standard assay procedures. Blood specimens were not processed in accordance with the recommendation of Pillemer *et al.*(2), and serum samples assayed had been stored in frozen state for approximately 1 year. The method of reading the tests was modified as a consequence of experience with repeatability. The results of experiments designed to test the importance of these variables will be presented below.

Results. Method of reading. Pillemer *et al.*(2) defined the unit of properdin as the least amount of serum which, in presence of an optimal amount of zymosan, *completely* inactivates 120 ± 30 units of C'3 in 1 ml of RP during 1 hour at 37°C. According to this definition, the end-point tube must show no hemolysis after addition of R3 and sensitized sheep erythrocytes. This criterion for the end-point has been modified slightly(4,5) to permit interpolation when minimal hemolysis is noted. Our experience with repeatability of the assay (Table I), resulted in adoption of a method of reading in which % hemolysis figures were arbitrarily divided into 3 groups: 0-30%, 40-70%, and 80-100%. Table I, in which units of properdin are read directly in terms of reciprocal of serum dilution, further explains the method of reading. In samples which exhibited a zone (Patient Cr, Table I), the tube which showed 50% hemolysis or less was taken as the end-point. Only 7 samples (4 from normal and 3 from splenectomized) fell into this category.

Method of processing blood samples. Pillemer *et al.*(2) centrifuged blood specimens at 2°C for 30 minutes at 4000 rpm, and then recentrifuged the serum to remove residual red cells. Samples we used were centrifuged 5-7 minutes at 2000-2200 rpm at room temperature. The effect of this difference in technic was tested by assaying 24 blood samples divided and processed by the 2 methods. These samples were drawn from patients visiting the University Hospital Hematology Clinic and varied in properdin content from 0 to 16 units/ml. The method of centrifugation had no effect on properdin content, in that the differences noted were well within the limits of repeatability.

Effect of age of serum sample. All serum samples from splenectomized subjects had been stored at –22°C for 11-13 months before being assayed. Other workers(5,6) utilized sera stored 6-7 months, and reported no change in properdin titer during this time. To our knowledge, longer periods have not been

† Kindly supplied by Dr. B. E. Sanders of Merck Inst. of Therapeutic Research.

TABLE I. Repeatability of the Zymosan Assay for Properdin.

Sample	No. of assays	% hemolysis: Reciprocal of serum dilution U	2	4	8	16	Properdin units
Rb	6*	0	0	0	0	100	8
	1	0	0	0	0	90	8
Ru	4	0	0	0	0	100	8
	1	0	0	0	0	90	8
	1	0	0	0	50	100	6
He	3	0	0	0	100	"	4
	1	0	0	0	90	"	4
	1	0	0	0	80	"	4
	3	0	0	0	70	"	6
	1	0	0	0	60	"	6
	1	0	0	0	50	"	6
Co	1	0	0	0	75	"	4
	2	0	0	0	50	"	6
	1	0	0	0	40	"	6
	1	0	0	0	35	"	6
	4	0	0	0	25	"	8
	2	0	0	0	20	"	8
	2	0	0	0	15	"	8
Ro	1	0	60	100	100	"	1½
	1	0	30	"	"	"	2
	1	0	"	70	"	"	2
	1	0	20	60	"	"	2
	1	0	10	100	"	"	2
	1	0	0	40	"	"	3
Cr	1	80	50	90	"	"	2
	1	70	30	80	"	"	2
	1	60	0	25	90	"	2

* Indicates No. of times assay results were as indicated to the right.

TABLE II. Effect of Storage at −22°C on Properdin Content of Normal Human Serum Samples.

Subject	Length of storage (mo) 13–14	11–12	8–10	2–6
	Units of properdin			
Fo	2	4	4	4
Do	12	16	12	16
Va	8	4	6	4
Up	6	6	8	8
Wh-1	6	3	4	4
Ra	16	8	16	8
Fe	8	8	4	8
Oc	8	12	16	16
Mo	0		0	0
Wh-2	0		2	1
Sa	16		12	12
Ca	8		12	12
Co	8		6	4
Lu	16		16	12
Wi	0		1	0
Sp	0		0	0
Re	8		12	8
Mu	0		0	0
	Mean = 6.78		Mean = 6.50	

Difference in means not significant (t = .47).

reported. The effect of age of serum sample on properdin content was controlled by us by assaying normal serum samples which had been stored for varying periods, and by including in the control group of 113 normal serum samples, 70 which had been stored for 11-13 months. The results of these studies, Tables II and III, indicate that serum samples can be stored for approximately one year with no significant change in properdin titer.

Properdin levels in splenectomized persons. Table IV presents results of properdin assays on serum specimens from 105 splenectomized and 113 normal human subjects. Mean titers for the 2 groups were 3.71 units and 6.35 units, respectively, and the difference between the 2 means was highly significant (t = 4.89). Thirty-seven (35.2%) of the samples from splenectomized individuals and only 8 (7.1%) from normals were without properdin. Data on the 105 splenectomized subjects analyzed by the multiple regression method(7) showed no correlation between properdin level and age, age at splenectomy, years post-splenectomy and underlying disease (as grouped in Table IV) for which splenectomy was performed. There was, however, a correlation between properdin level and sex in that titers were significantly lower in females. This difference, significant at the 5% level but not at the 1% level, was a characteristic of the entire group of 105 patients and, to a varying extent, of each of the 5 sub-groups in Table IV. The greatest difference was noted in the congenital hemolytic anemia group. Normal males and females exhibited comparable properdin titers(8).

There was no correlation between level of properdin and ability to produce antibodies after subcutaneous inoculation of tularemia vaccine(1). Peak antibody titers were not significantly different in 53 subjects with low properdin (2 units or less) and in 52 subjects with normal amounts. Rate of formation of antibody was similar in the 2 groups in that mean antibody titers were not significantly different 1 week after immunization. Lack of correlation between properdin level and antibody production was obvious in 6 subjects splenectomized because of traumatic rupture of the spleen. All of these patients produced antibody in normal amounts(1) whereas 5 of 6 showed no properdin.

The above comparison of properdin titer and antibody production in the 105 splenectomized subjects does not take into consideration the slight but significant sex difference in properdin level. This difference takes on added importance in light of the fact that 11

TABLE III. Properdin Levels in "Fresh" and Stored Normal Human Serum Samples.

Storage (mo)	No. of samples	Properdin units 0	1	2	3	4	6	8	12	16
0- 2.5	41	1	2	4	3	5	4	15	5	2
11-13	72	7	3	5	3	10	8	28	5	3
			Low <3			Normal 3-12				High 16
0- 2.5	41		7-17%			32-78%				2-5%
11-13	72		15-21%			54-75%				3-4%

Mean properdin titer, "Fresh"—6.71. Mean properdin titer, Stored—6.14.
Difference in means not significant (t = .75).

TABLE IV. Properdin Levels in Splenectomized and Normal Persons.

Diagnosis	No. of subjects	Properdin units 0	1	2	3	4	6	8	12	16	Mean titer
Congenital hemolytic anemia	23	9	1	3	1	1	2	5	1		3.39
Idiopathic thrombocytopenic purpura	37	9	1	7	3	8	2	5	1	1	3.68
Acquired hemolytic anemia	13	3	1	2	2			4		1	4.54
Secondary thrombocytopenic purpura	10	3	1			3		3			3.70
Primary splenic neutropenia	6	2				1		2	1		
Secondary " "	2					1			1		
Primary splenic panhematopenia	2							1		1	
Secondary " "	3	3									3.64
" hemolytic anemia	2	2									
Aplastic anemia	1	1									
Traumatic rupture	6	5						1			
Total splenectomized	105	37	4	12	6	14	4	21	4	3	3.71
" normals	113	8	5	9	6	15	12	43	10	5	6.35

		Low <3 units	Normal 3-12 units	High 16 units
Total splenectomized	105	53-50%	49-47%	3-3%
" normals	113	22-19%	86-76%	5-4%

of 70 females and only 2 of 35 males did not produce antibodies after tularemia vaccination(1). These observations are not necessarily indicative of any immunologic weakness in splenectomized females but further studies are suggested.

Discussion. Our results demonstrate that properdin levels in splenectomized persons are lower than in normal persons. Although a minor sex difference was noted in the group of splenectomized patients studied, there was no correlation between properdin level and age of the individual, age at splenectomy, years post-splenectomy, or the underlying disease for which splenectomy was performed, leaving absence of the spleen the most probable common characteristic. Admittedly, the heterogeneous nature of some of the sub-groups in Table IV detracts somewhat from this contention. However, the fact that 5 of 6 subjects splenectomized because of traumatic rupture of the spleen showed no properdin is of significance.

The sex difference in properdin level in the splenectomized group was minimal in comparison to the difference between properdin levels in the splenectomized and control groups. Properdin titers were lower in females, however, and this observation is of interest in that 11 of 70 females and only 2 of 35 males did not produce antibodies after tularemia vaccination(1). In general, however, there was no correlation between properdin level and ability to produce antibodies. All but 13 of 105 splenectomized subjects produced antibodies(1), whereas about half (53 of 105) showed little or no properdin. An attractive but purely speculative interpretation of these facts would be that the spleen is one of the principal sites of properdin formation and, after splenectomy, other organs take over this function of the spleen to a lesser and more variable degree than they do the antibody-forming function of the spleen. The data in the present report do not warrant such an interpretation, however, since they do not prove conclusively that the low properdin levels were due to absence of the spleen rather than to the underlying disease process. Our present knowledge of the properdin system and the *in vivo* activities which seem to affect properdin levels as determined by the zymosan assay make it reasonable to believe that subclinical conditions or altered physiologic states could be responsible for the observed low properdin values. However, if this were the case, these factors would have to be operative to the same degree in all of the disease sub-groups in Table IV, since the means of the different sub-groups were not significantly different. Further studies are being carried out in this laboratory in an attempt to clarify this point.

Summary. Properdin titers were definitely lower in 105 splenectomized persons than in 113 normal, healthy subjects. Within the

splenectomized group, there was no correlation between properdin level and age of individual, age at splenectomy, and years post-splenectomy. Properdin titers were significantly lower in splenectomized females, but the sex difference was minimal in comparison to the difference between total splenectomized and control groups. Suggestive evidence is presented that low properdin levels in splenectomized persons are related in part to absence of spleen.

The authors are indebted to Joann Sparks for technical assistance and to Dr. D. R. Whitney, Director of Statistics Research Lab., for the more complex statistical analyses.

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Received July 16, 1959. P.S.E.B.M., 1959, v102.

Trypsin, Invertase and Amylase Content of Feces of Germfree Rats.* (25174)

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As measured with specific methods the trypsin and chymotrypsin concentration of intestinal content decreases with length of the intestine, decreasing to zero in feces(1). This fact indicates that intestinal enzymes are autodigested or are inactivated by intestinal microorganisms. To elucidate this problem the enzyme content in feces of germfree and conventional animals was measured.

Material and methods. Germfree rats reared according to Gustafsson(2-3) were used in these studies. Two germfree male adult rats of the 4th generation were fed the semisynthetic diet D_6(4) and kept individually in metabolism cages. Feces and urine were collected each 24 hours. Two conventional rats were fed the same autoclaved diet and kept in metabolism cages in the animal room. The feces were collected for 8 consecutive days. On the 8th day the germfree rats were infected with cecal content of a conventional animal heated to 90°C for 5 min. Thereafter the feces were collected for another 12 days during which time sporeforming organisms were cultivated from the feces. Then one of the animals was killed for assessment of the effect of the sporeformers on the cecum enlargement. The remaining germfree animal was on the same day infected in the apparatus with a suspension of feces from the conventional rats. The feces were homogenized with 10 ml ice cold saline and centrifuged. Aliquots of the clear supernatant solution were used for enzyme determinations. Trypsin was determined spectrofotometrically using benzoyl-arginine ethyl ester as substrate(5). Invertase activity was assayed according to a modification of the method of Sumner(6), and expressed as mg of invert sugar produced in one hour by 1 ml of the sample at 25°C. Amylase activity was determined by the method of Meyer *et al.*(7) and expressed as mg of maltose liberated in 3 minutes by 1 ml of the sample at 25°C.

Results. The feces of the conventional animals did not contain measurable amounts of trypsin or invertase but held 40-100 units of amylase (Fig. 1). On the other hand, feces of the germfree rats contained 1-6 mg trypsin daily and 12-25 units of invertase per day.

* This investigation supported by grants from Swedish Medical Research Council, Wallenberg Fn., Stockholm, Sweden, and Nat. Inst. Health, Bethesda, Md.

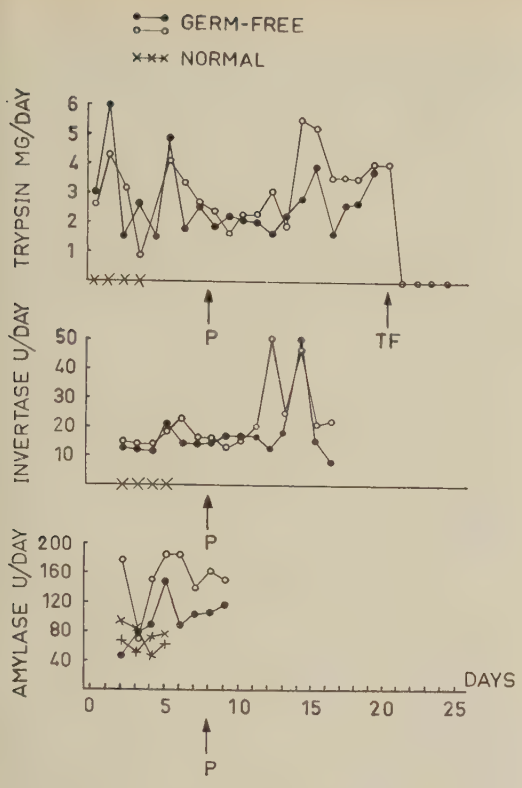

FIG. 1. Trypsin, invertase and amylase content of feces of germfree, ex-germfree and conventional rats. At P germfree animals were infected with sporeforming bacteria from the cecum of a conventional animal and at TF with suspension of feces from conventional rats.

Amylase content of the feces in the germfree rats was the same or slightly higher than that of the conventional rats. The occurrence of invertase in the feces of germfree animals agrees with the finding of carbohydrases in intestinal extracts from germfree rats(8). After infection of the germfree animals with sporeforming bacteria from the cecum content of a conventional animal, no decrease occurred in the enzymatic contents of the feces but rather sharp fluctuations were noticed in invertase and trypsin activity. This might be connected with the diarrhea that is a constant symptom in infection of germfree animals with certain intestinal bacteria. After superinfection with full intestinal flora of the conventional animals, tryspin content of the feces decreased to the zero values of the conventional rats within 24 hours.

Conclusions. Results indicate that autodigestion is not the main pathway for normally occurring inactivation of trypsin and invertase. The results infer that one or several of the normal microbiological inhabitants of the intestines are responsible for normally occurring inactivation of the digestive enzymes of intestinal contents.

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Received July 17, 1959. P.S.E.B.M., 1959, v102.

Effect of Dietary Fat on Excretion and Deposition of C^{14} from Cholesterol-4-C^{14} in Rats.* (25175)

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The relationship between serum cholesterol levels and dietary factors has been studied in man and lower mammals for many years. Recently it has become important to ascertain

* This investigation supported by grants from Life Insurance Medical Research Fund, U.S.P.H.S., and American Med. Assn.

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TABLE I. Fecal Excretion of C^{14} by Rats Fed Crisco, Corn Oil, or Coconut Oil and Injected with Cholesterol-4-C^{14}.

Dietary fat	No. of animals	Fecal excretion of C^{14} (% of administered dose) At 4 days Mean	S.D.*	At 14 days Mean	S.D.*
Crisco	10	13.7	4.6	51.9	7.6
Corn oil	10	11.6	3.2	51.7	7.6
Coconut oil	7	14.4	7.9	52.9	11.8

* Stand. dev.

the fate of serum cholesterol in those instances in which serum concentration has been influenced by specific dietary alterations. Evidence that cholesterol lost from human serum when unsaturated fats are added to the diet is excreted in some form was obtained by Hellman and coworkers(1) and by Gordon *et al.* (2). Wilson and Siperstein(3,4) noted no consistent differences in total recovery of fecal C^{14} by rats injected with cholesterol-4-C^{14} when fed 20% corn oil or 20% lard diets. We have studied the fecal excretion of C^{14} and incorporation of C^{14} in tissue lipids of rats fed diets containing corn oil, coconut oil, or hydrogenated cottonseed oil and injected with cholesterol-4-C^{14}.

Materials and methods. Male Sprague-Dawley rats weighing 75-100 g were placed on a purified diet‡ containing 20% fat. The fats used were: hydrogenated cottonseed oil (Crisco, iodine No. 73), corn oil (Mazola, iodine No. 123), and coconut oil (iodine No. 10). The rats were paired by weight and those in the group receiving corn oil or coconut oil fed the amount of food consumed by the corresponding rat on the Crisco diet the preceding day. A solution of cholesterol-4-C^{14} containing 1 mg and 680,000 cpm per ml was prepared by the use of Tween-20 according to the method of Meier *et al.*(5). After the rats had been on the experimental diets for 2 to 3 weeks 0.5 ml of the cholesterol solution was injected into the external jugular vein. Feces were collected at 1, 2, 4, 6, 10, and 14 days. The animals were then killed by decapitation, livers removed and placed immediately in 10% alcoholic KOH. The heart, lungs, aorta, and kidneys of each animal were pooled and treated as one sample with alcoholic KOH. After hydrolysis the unsaponifiable material was extracted with petroleum ether. Fecal samples were extracted using the method outlined by Siperstein and coworkers(6). Radioactivity determinations were made on all extracts by gas flow counting of direct mounts prepared according to the method of Entenman *et al.*(7). Corrections were applied for background and self-absorption. Serum total cholesterol determinations were done by the method of Sperry and Webb(8).

Results. The per cent of administered dose of C^{14} excreted in feces by the 3 groups of rats is given in Table I. Similar quantities were excreted by the 3 groups at all time periods studied and approximately 52% of the administered dose was excreted in 14 days.

Analysis of C^{14} in extracts of total nonsaponifiable material in liver and in a pooled sample of heart, lungs, aorta, and kidneys revealed no significant differences in the 3 groups of rats (Table II).

No significant difference in serum cholesterol values of the 3 groups of rats resulted from feeding the various fats for a period of 4 to 5 weeks.

Discussion. These studies indicate that after 4 to 5 weeks on a purified diet containing 20% fat as hydrogenated cottonseed oil, corn oil, or coconut oil there is no difference in amount of C^{14} excreted in feces or found in livers or in a pooled sample of heart, lungs, kidneys and aorta in rats injected with cholesterol-4-C^{14}. In man there appears to be increased excretion of cholesterol derivatives

TABLE II. Amount of C^{14} in Tissues of Rats Fed Crisco, Corn Oil, or Coconut Oil and Injected with Cholesterol-4-C^{14}.

Dietary fat	% of administered dose Liver Mean	S.D.	Pooled organs* Mean	S.D.
Crisco	2.31	.50	1.86	.41
Corn oil	2.80	.65	1.85	.27
Coconut oil	2.21	.53	1.69	.41

* Heart, lungs, aorta, and kidneys.

‡ Vitamin-free casein, 250 g; fat, 200 g; cod-liver oil, 20 g; sucrose, 480 g; vitamin supplement mixture with sucrose, 21 g (this mixture contained choline hydrochloride, 0.5 g; thiamine hydrochloride, 2.5 mg; pyridoxin, 2.5 mg; inositol, 1 g; calcium pantothenate, 30 mg; riboflavin, 5 mg); salt mixture (Mendel, Hubbell and Wakeman), 31 g.

when unsaturated fats are added to the diet (1,2), but Wilson and Siperstein(3,4) found no consistent difference in fecal excretion of C^{14} by rats fed 20% corn oil or 20% lard and injected intravenously with cholesterol-4-C^{14}. Both groups, however, excreted a larger amount of injected C^{14} than did rats fed a fat-free diet. The amount of C^{14} in non-digitonin-precipitable sterols was greatly increased in animals on the corn oil diet as compared to rats receiving either no fat or a 20% lard diet. In rats fed these fats at 30% of the diet the latter observation was confirmed and in addition, it was noted that in 4 out of 5 groups total C^{14} recovery in feces was greater in rats receiving corn oil. In our experiments using 20% fats in the diet the rats excreted about 52% of administered C^{14} in 14 days. This compares favorably with data of Wilson and Siperstein(3,4) in the experiments using fat at 20% of the diet. Fractionation of the C^{14} excreted in the feces was not included in our experiment. It is apparent that in these rats the effect of kind of fat fed is on type of sterol excreted rather than on total amount.

In studies of the influence of dietary fat on serum cholesterol levels it is necessary to ascertain the possible simultaneous effect of the diet on tissue deposition of cholesterol. Alfin-Slater *et al.*(9) found an accumulation of cholesterol in livers and adrenals of rats on an essential fatty acid-deficient diet with a corresponding decrease in serum cholesterol levels. Addition of essential fatty acids to the diet caused a reversal of this situation. The data presented in Table II indicate that under the conditions of our experiments varying the type of fat in the diet for a 4- to 5-week period did not influence amount of C^{14} deposited in livers or pooled organs (heart, lungs, aorta, and kidneys) after intravenous injection of cholesterol-4-C^{14}.

Summary. The influence of a purified diet containing 20% fat as hydrogenated cottonseed oil, corn oil, or coconut oil on fecal excretion and tissue deposition of C^{14} has been studied in rats injected with cholesterol-4-C^{14}. There was no significant difference between the 3 groups in amount of C^{14} excreted in feces or deposited in liver and in pooled internal organs (heart, lungs, aorta, and kidneys).

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Received July 20, 1959. P.S.E.B.M., 1959, v102.

Polarographic and Biologic Activities of N-Substituted Maleimides.* (25176)

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Friedman, Marrian and Simeon-Reuss(1) discovered that the maleimide molecule has antimitotic properties and forms –SH adducts readily. Recently(2) N-ethylmaleimide has been found to react with a number of thiols and thiol esters and the reaction

* Investigation supported by grant G-45 from Nat. Science Fn.

† The author gratefully acknowledges advice and guidance of Dr. Stanley Wawzonek in measuring half-wave potentials and the technical assistance of Dr. Leslie Paleg.

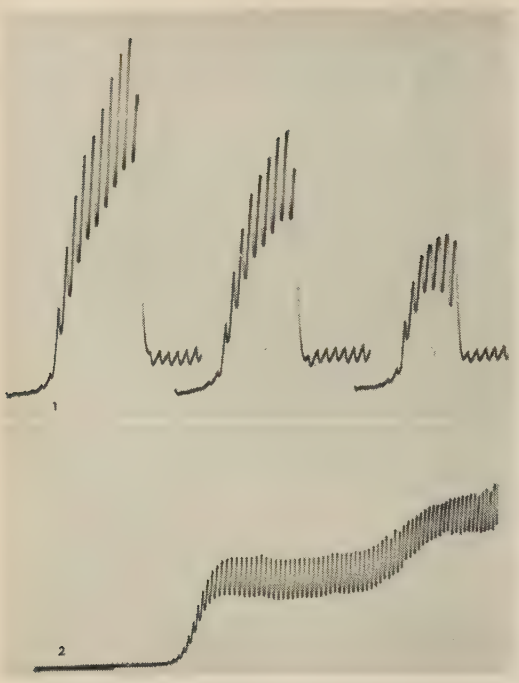

FIG. 1. Polarogram of oxygen maximum in the electrolyte solution without maleimide (left) and oxygen maxima following additions of one and 2 aliquots (5 μl) of 5×10^{-3} M N-phenylmaleimide.

FIG. 2. Polarogram of reduction of N-(2,4-dichlorophenyl)maleimide. Ordinate: current. Abscissa: voltage.

has been used in chromatography of such compounds. In examining defoliation and growth effects of sulfhydryl reagents van Overbeek, Blondeau and Horne(3) employed substituted maleimides and reported that chlorination of the ring of N-phenylmaleimide increased its effect in inhibiting growth, and saturation of the double bond of the maleimide ring abolished such effects. The increase in activity due to chlorination of the ring was attributed to increase in lipoid solubility favoring penetration through cell membranes. It is also possible that reactivity of the double bond is affected by substitution in the phenyl ring and inhibition of growth is related to ease with which addition of –SH to the unsaturated system takes place. Wawzonek and Fossum (4) found the ease of reduction of unsaturated carbonyl system of esters and amides of β-aroylacrylic acid as measured by the dropping mercury electrode, parallels the antibacterial activity of the compounds. Reduction of maleimides at the dropping mercury electrode shows that substituents in the phenyl ring do affect reactivity of the maleimide double bond.

Materials and methods. Polarograms were obtained with a Sargent Model XII Recording Polarograph. With increasing applied e.m.f. in presence of oxygen an increased reduction current flow is obtained, reaching a maximum (oxygen maximum) in these determinations at –0.4 volt and then dropping sharply to the diffusion current level of the electrolyte solution (Fig. 1). The effects of maleimides‡ upon oxygen maximum were measured in 10 ml of 0.01 N KCl with 8% methyl alcohol. Aliquots of maleimide dissolved in electrolyte-methyl alcohol solution were added to the electrolysis vessel until the maximum was depressed to less than one-half of its value in the original electrolyte solution. The drop time was 7.2 seconds with bath temperature of 24°C. Shunt ratio was 50 and span electromotive force was 4 volts. Amount of maleimide required to depress oxygen maximum by exactly one-half is the half-suppression value (H.S.V.) for the compound in micromoles/liter. Reduction of an organic molecule at the dropping mercury electrode gives rise to a "polarographic wave" in the current-voltage measurement (Fig. 2). Standard practice reports polarographic reduction potential as the potential of the midpoint of the wave where the current is equal to half its limiting value. This potential is termed the half-wave potential ($E_{1/2}$) and is referred to the saturated calomel electrode at 25°C. Half-wave potentials for maleimides were measured in a solution of 0.1M tetrabutylammonium iodide and 0.001% methyl red in 50% *p*-dioxane by volume. Dioxane was freshly purified. Maleimide concentration was 4 millimolar, drop time was 4.3 seconds, shunt ratio was 50 and span electromotive force was 2 volts. Electrolysis vessels were gassed with nitrogen for 30 minutes before obtaining the current-voltage curves. Biologic activity of maleimides in repressing elongation of cells was determined by a standardized procedure in which growth of 3 mm segments of Avena coleoptile tissue induced

‡ Purified samples kindly supplied by Dr. J. van Overbeek of Shell Development Co.

TABLE I. Half-Suppression Values, Half-Wave Potentials and Growth Inhibition for N-Substituted Maleimides.

Compound	H.S.V.,* μmole/l	$-E_{½}$ (S.C.E.),† v	% inhibition of IAA-induced growth by maleimide‡ IAA 2×10^{-7}M	IAA 2×10^{-5}M
N-Isopropylmaleimide	>110	.89	1	1
N-Phenylmaleimide	3.9	.80	25	15
N-(4-Chlorophenyl) maleimide	1.8	.76	35	20
N-(2,4-Dichlorophenyl) maleimide	.55	.76	65	50
N-(2,4,6-Trichlorophenyl) maleimide	.55	.74	100	100

* Amount of maleimide required to reduce maximum current flow due to oxygen by one-half.
† Half-wave potential with reference to saturated calomel electrode.
‡ Maleimide concentration 5×10^{-5}M in all cases.

by indole-3-acetic acid (IAA) in 0.8% isopropyl alcohol solutions was measured after 12 hours.

Results. The effects of substances upon the oxygen maximum may be used as a relative measure of their adsorbability or surface activity (5), a small H.S.V. thus indicates high adsorbability or surface activity. Within the series of maleimides in Table I there is an extreme variation in H.S.V., the value for the N-isopropyl compound being very large while di- and trichloro- compounds have values only a little larger than the lowest value yet obtained (0.3 for 4-chloroindole-3-acetic acid). Water solubility of these compounds (3) varies in the same direction as surface activity but the magnitude of variation in solubility is at least 5 times greater (N-isopropyl- >5,000 ppm, N-phenyl- 75-100 ppm, N-(4-chlorophenyl)- and N-(2,4-dichlorophenyl)- 5 ppm.

No reduction waves for N-phenylsuccinimide at potentials less than –2.0 v S.C.E. were found and the waves recorded for maleimides therefore represent reduction of the carbon-carbon double bond. Heights of waves (I_d = 0.85 and 0.5 for N-(2,4-dichlorophenyl) maleimide, Fig. 2) point to a 1-electron change for each wave. The half-wave potential of the first wave, measures the relative ease of electron additions to the double bond and reflects electrical and steric effects present in the molecule. Addition of RS^- would be subject to similar effects. The half-wave potential of the second wave would have no significance for the biologic addition reaction but was –1.49, –1.3, –1.25, –1.3, and –1.32 v respectively, for compounds listed in Table I. The values of half-wave potentials for the first wave show that the chemical group on the N-atom of the maleimide structure affects reduction of the double bond with the aromatic ring increasing ease of reduction considerably and the 2,4,6-trichloro-substitution resulting in maximum effect.

Growth effects of maleimides at 5×10^{-5}M concentration are recorded in Table I. At this concentration N–(2,4,6-trichlorophenyl) maleimide prevents completely the growth that may be induced by the growth hormone, indole-3-acetic acid (IAA). N-isopropylmaleimide at this concentration has a minimum perceptible effect represented by 1% inhibition. With higher concentrations of the maleimides, inhibition is complete even for N-isopropylmaleimide (4×10^{-4}M).

Discussion. Explanations of differences in biologic activity of a group of analogs such as the series of maleimides examined usually emphasize solubility or lipophilic characteristics rather than chemical reactivity. Since little is known of the functional significance of the former and the latter is usually entirely unknown the arbitrary choice cannot be criticized. Even where information is more complete the basis for activity as related to structure may be obscure. For the 5 maleimide compounds the differences in biologic activity correspond generally to differences in water solubility, lipophilic properties, adsorbability or surface activity, and ease of reduction at the maleimide carbon-carbon double bond. However, this correspondence simply relates the several properties as codependent on molecular constitution.

The most effective compound, N–(2,4,6-trichlorophenyl)maleimide, is virtually insol-

uble in water although very dilute solutions may be prepared by first dissolving the compound in a small quantity of isopropyl alcohol. Since N-isopropylmaleimide is more than 5,000 times as soluble in water, yet causes 100% inhibition of growth at only an 8-fold increase in concentration, it is unlikely that oil/water solubility properties are responsible for the differences in inhibition. Similarly the considerable difference in the H.S.V. of the isopropyl compound compared to those of the most effective compounds indicates little dependency of inhibition effects on adsorbability or surface activity.

The chemical reactivity of maleimides appears the logical basis for their biologic activity. The molecular configuration which can add –SH more readily at the carbon-carbon double bond is more effective as inhibitor of the growth response. Thus, N–(2,4,6-trichlorophenyl)maleimide with the most positive half-wave potential is the most effective inhibitor. A half-wave potential more negative by 20 millivolts results in less inhibitory effect and the least inhibitory effect is given when the half-wave potential is more negative by 150 millivolts. The fact that the same half-wave potentials were obtained for mono- and di-chloro-compounds may be attributed to limitations of the method in measuring potential differences as small as 10 millivolts. It is also possible that with the same half-wave potential the greater inhibition by the dichloro-compound as compared with N–(4-chlorophenyl)maleimide results from its greater adsorbability or surface activity. Yet the more important effect of chemical reactivity is demonstrated by the dichloro- and trichloro-compounds having the same H.S.V. but different half-wave potentials and different degrees of inhibition.

Some indications of a specificity of maleimides for sulfhydryls involved in the growth process were obtained by van Overbeek, *et al.* (3) when they found that 5×10^{-5} M N–(2, 4-dichlorophenyl) maleimide inhibited growth by 50% while reducing respiration only 20% and N-isopropylmaleimide inhibited growth 23% and respiration only 5%. Such specificity, if real, would have its foundation in critical chemical reactivity as indicated by measurements of the half-wave potentials.

Summary. The half-suppression values and half-wave potentials for 5 N-substituted maleimides were measured with the polarograph and compared with their inhibitory effects on growth of plant tissue induced by indoleacetic acid. The chemical group at the N-atom of the maleimide structure determines the ease of reduction of the carbon-carbon double bond. This chemical reactivity appears to be the basis for the biologic activity of maleimides with some possibility of adsorbability or surface activity playing a minor role.

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Received March 24, 1959. P.S.E.B.M., 1959, v102.

Anaphylactic Shock in Mouse II. Allergenicity of Water-in-Oil Emulsions With or Without *Mycobacterium.** (25177)

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Our previous reports(1,2) have shown successful production of severe or fatal anaphylaxis in the CFW mouse sensitized with a single dose of bovine albumin incorporated into water-in-oil emulsion containing killed *Mycobacterium butyricum*. This experimental model was employed in studying effects of whole-body x-irradiation(2) on hypersensitive state and response of mice. These earlier studies revealed that anaphylactic sensitivity was lacking in animals challenged intravenously with bovine albumin 1,3,5, or 7 days postsensitization. Challenge of mice 10, 14, 21, or 28 days after sensitization killed 15, 16, 43, and 67% respectively. Pooled antiserum secured from mice 28 days postsensitization conferred passively fatal anaphylactic sensitivity to normal mice; this antiserum contained precipitins for antigens in both bovine albumin and bovine gamma globulin(2,3). This report presents evidence that under certain conditions a water-in-oil emulsion lacking *Mycobacterium butyricum* (incomplete adjuvant) may be as effective in potentiating anaphylactogenicity of bovine albumin in mice as one containing acid-fast bacilli (complete adjuvant). We report determination of minimal challenging dose of bovine albumin in mice sensitized with protein combined with complete adjuvant. Data are included to show duration of anaphylactic sensitivity in mice injected with bovine albumin incorporated into the complete adjuvant.

Materials and methods. CFW female mice between 4 to 6 weeks of age at time of sensitization were employed. The allergen used in sensitizing and challenging procedures was bovine albumin employed similarly in earlier studies(1,2). Bacto-adjuvant, complete (Freund) used in our earlier studies(1,2) and Bacto-adjuvant, incomplete (Freund) were employed in preparing separate water-in-oil emulsions containing bovine albumin. The incomplete adjuvant had the same composition as the complete one except that the former contained no *Mycobacterium butyricum*. Method of preparation of bovine albumin-adjuvant emulsions was described previously(1, 2). Details of sensitizing and challenging procedures have been given(1,2). For sensitization each mouse received subcutaneously 0.2 ml of water-in-oil emulsion containing 0.1 mg of bovine albumin. The challenging dose of bovine albumin, contained in 0.2 ml of 0.85% NaCl solution, was injected rapidly into tail vein. Deaths were recorded at 60 minutes. Measurements of rectal temperature were made immediately before administration of challenging dose and in survivors 30 minutes following injection. Rectal temperatures were measured by thermocouple device as described previously(1,2).

Results. Table I gives results of experiment *to determine minimal challenging dose of bovine albumin* in mice sensitized with 0.1 mg of protein incorporated into *complete* adjuvant. Challenging doses (2.0, 0.2, or 0.1 mg) elicited death or severe signs of anaphylaxis in sensitized mice. Survivors failed to show signs of anaphylaxis when challenged again after 24 hours with bovine albumin. Smaller challenging doses (0.05, 0.02, or 0.01 mg) resulted in lower mortality incidences. Dose of 0.005 mg produced only minimal signs of anaphylaxis. Survivors given challenging doses less than 0.1 mg exhibited marked anaphylactic reactivity when rechallenged 24 hours later with bovine albumin.

Table II includes results of experiment concerning *duration of anaphylactic sensitivity* in mice injected with bovine albumin admixed with *complete* adjuvant. A high level of sensitivity was maintained between 35 and 84

* This study supported by contract with School of Aviation Medicine, USAF, Randolph Air Force Base, Texas.

† Present address: Community Blood Bank, Kansas City, Mo.

TABLE I. Determination of a Minimal Challenging Dose of Bovine Albumin for Sensitized Mice.*

Challenging dose, mg bovine albumin†	Challenge: Mortality	Challenge: No. of survivors at 30 min.	Challenge: $\triangle t_{30}$§	Rechallenge†: Mortality	Rechallenge†: No. of survivors at 30 min.	Rechallenge†: $\triangle t_{30}$§
2.0	8/10‡	3	−4.9	0/2	2	.0
.2	"	4	−5.2	"	2	+ .2
.1	8/15	10	−4.0	0/7	7	− .2
.05	3/10	7	−1.3	0/7	7	−3.1
.02	1/15	14	−3.1	5/13	10	−2.8
.01	2/15	14	−2.3	3/13	11	−3.0
.005	0/15	15	−1.2	5/15	12	−3.9

* Each mouse inj. with single sensitizing dose (0.2 ml) of bovine albumin-adjuvant emulsion containing 0.1 mg of bovine albumin.

† Challenging dose contained in 0.2 ml was inj. rapidly into tail vein 28 days following sensitization. Survivors were rechallenged 24 hr later with 0.2 ml of 1% bovine albumin in 0.85% NaCl solution.

‡ Numerator = No. of deaths recorded at 60 min.; denominator = No. of mice challenged.

§ $\triangle t_{30}$ = Mean change in rectal temperature (°C) of mice surviving 30 min. after challenge.

days. Level of sensitivity of mice tested after 84 days was much less than that of those challenged 35 to 84 days postsensitization. Diminished, but definite residuum of anaphylactic sensitivity was present 196 days postsensitization.

Table III contains results of experiment designed *to compare efficacy of complete and incomplete adjuvant* in enhancing allergenicity of bovine albumin in mice. Animals challenged 7 days postsensitization failed to exhibit signs of anaphylactic shock. Existence of sensitized state was evident after 10 days when either the complete or incomplete adjuvant was employed in the sensitizing mixture. When challenges were made 14, 21, 28, or 35 days postsensitization, mortality incidences in mice receiving complete adjuvant was insignificantly different from that in animals receiving incomplete one.

TABLE II. Duration of Anaphylactic Sensitivity in Mice Injected with Bovine Albumin-Adjuvant Emulsion on 9 Groups.

Days between sensitization* and challenge†	Incidence of mortality	Survivors at 30 min.	$\triangle t_{30}$§
35	10/15 (.67)‡	7	−5.0
42	25/27 (.93)	7	−5.4
49	14/15 (.93)	4	−6.1
56	11/15 (.73)	6	−6.1
84	15/36 (.42)	27	−2.8
112	0/15 (0)	15	−2.8
140	" (0)	15	−2.7
168	4/15 (.27)	11	−2.4
196	1/9 (.11)	8	−3.3

* Each mouse inj. with single sensitizing dose (0.2 ml) of bovine albumin-adjuvant emulsion containing 0.1 mg of bovine albumin.

† Challenging dose (0.2 ml) consisting of 1% bovine albumin in 0.85% NaCl solution inj. into tail vein.

‡ Numerator = No. of deaths recorded at 60 min.; denominator = No. of mice challenged; No. in parentheses is decimal fraction.

§ $\triangle t_{30}$ = Mean change in rectal temperature (°C) taken 30 min. after challenge with bovine albumin.

Discussion. In our work, no attempt was made to determine the effect of *M. butyricum* in water-in-oil emulsion on antigenicity of bovine albumin in mice; precipitin determinations were not made. Furthermore, skin tests were not done to determine development of sensitivity of the delayed type. However, our results did show that anaphylactogenicity of bovine albumin in water-in-oil emulsion was not enhanced significantly by addition of *M. butyricum,* at least under conditions of experiments employed. Consequently for studies of anaphylactic shock in mice, the incomplete adjuvant may be employed as a suitable potentiating agent of sensitization. In progress are experiments to contrast precipitin levels in sera of mice sensitized with bovine albumin admixed with complete or incomplete adjuvant.

Our experiments revealed that mice sensitized with 0.1 mg of bovine albumin emulsified with the complete adjuvant demonstrated signs of anaphylactic shock when challenged with bovine albumin as long as 196 days postsensitization.

Summary. 1) Mice sensitized with 0.1 mg

TABLE III. Comparison of Complete and Incomplete Adjuvants in Their Ability to Induce Anaphylactic Sensitivity in Mice to Bovine Albumin.

Days between sensitization* and challenge†	Adjuvant in sensitizing emulsion*	Incidence of mortality	Survivors at 30 min.	$\triangle t_{30}$§
7	Complete	0/20 (0)‡	20	+ .2
7	Incomplete	" (0)	"	+ .6
10	Complete	1/25 (.04)	24‖	−3.3
"	Incomplete	0/25 (0)	25	−1.1
14	Complete	9/26 (.35)	18	−2.1
"	Incomplete	10/18 (.55)	9	−2.8
21	Complete	11/24 (.46)	16	−4.4
"	Incomplete	13/25 (.52)	15	−5.6
28	Complete	59/89 (.66)	38	−3.5
"	Incomplete	58/89 (.65)	39	−3.4
35	Complete	22/24 (.92)	11	−7.1
"	Incomplete	19/21 (.90)	5	−7.8

* Each mouse inj. on same day with a single sensitizing dose (0.2 ml) of bovine albumin-adjuvant emulsion containing 0.1 mg of bovine albumin.

† Challenging dose (0.2 ml) consisting of 1% bovine albumin in 0.85% NaCl inj. rapidly into tail vein.

‡ Numerator = No. of deaths recorded at 60 min.; denominator = No. of mice challenged; No. in parentheses is decimal fraction.

§ $\triangle t_{30}$ = Mean change in rectal temperature (°C) of mice surviving 30 min. after challenge.

‖ Two of the 24 died within 3 hr following challenge.

of bovine albumin incorporated into water-in-oil emulsion containing *Mycobacterium butyricum* showed anaphylactic reactivity when challenged 28 days postsensitization with as little as 0.01 mg of bovine albumin. 2) Mice sensitized in a similar manner exhibited signs of anaphylaxis when challenged 196 days following sensitization. 3) Anaphylactogenicity in mice of bovine albumin in emulsions lacking *Mycobacterium butyricum* was comparable to that in emulsions containing acid-fast bacilli.

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Received May 15, 1959. P.S.E.B.M., 1959, v102.

Anorexigenic Action of Methylphenidate (Ritalin) and Pipradrol (Meratran). (25178)

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Methylphenidate (Ritalin) and pipradrol (Meratran) are recently developed piperidine derivatives with central nervous system stimulant action. Meier *et al.*(1) and Brown *et al.* (2) suggested that the piperidine stimulants differ in certain respects from excitants of the amphetamine type, but they also demonstrated that these two classes of compounds share many pharmacologic properties. However, the newer agents have not been tested for possible anorexigenic action, so characteristic clinically and pharmacologically for drugs of the amphetamine class(3). Accordingly, an antiappetite assay was set up in experimental animals in order to compare piperidine stimulants with amphetamine-like agents.

TABLE I. Anti-appetite Index (AAI) following Subcutaneous Administration of Piperidine- and Amphetamine-Class Agents.

	AAI expressed in %						
	Dose, mg/kg						
Agent	.126	.25	.33	.40	.63	1.0	1.58
Methamphetamine HCl (d-desoxyephedrine)	9	15	20	29	56	61	
d-amphetamine SO_4	15	21	29	32	62	72	
Ritalin HCl (methylphenidate)		11		20	23	49	78
Pipradrol HCl (Meratran)	6	15	20	25	59		69

The results are reported here.

Methods. Anorexigenic testing was carried out in cats selected and trained to develop constancy of eating habits. Each drug was administered subcutaneously at not less than 4 logarithmically spaced dose levels; 5 cats were employed per dose level. Each dose was given 3 times, 45 minutes prior to and 6 and 16 hours following feeding time; medication was discontinued thereafter. Time elapsed between presentation of food and first food consumption was measured and averaged in each group of 5 cats. This average was referred to as average eating latency (AEL). Control animals eat as soon as the food is presented; in these, AEL is zero. Food consumption was recorded over 3-day periods in treated and untreated animals. In 150 controls variability of food consumption was not more than 10%; similar variability was found in the case of medicated animals. The difference between average food consumption of experimental and control cats was expressed in per cent of control value and referred to as average anti-appetite index (AAI). Minimal effective dose (MED) was the average dose capable of producing an AEL of 30 minutes or/and AAI of at least 20%. In some instances the method of Estes and Skinner(4) involving operant behavior of rats was adopted to anorexigenic screening. Rats were trained either solely in bar pressing for aperiodic food reward, or conditioned additionally to interrupt their bar pressing behavior at the onset of a click-shock sequence. These latter animals are said to develop conditioned emotional response (CER) or "anxiety"(5). After a suitable training period the drugs were administered in single doses at 2 dose levels *via* subcutaneous route, 2 to 3 rats per dose. Effect on coordinated motor activity was measured in rats by the method of Schulte *et al.*(6). Each agent was given at 3 to 4 logarithmically spaced dose levels, 5 to 6 rats per dose. The MED values were estimated from the dose-effect curves as the points of occurrence of a 30% increase in activity. LD_{50} values were obtained in mice by the method of Miller and Tainter(7); in cats, approximate toxicities (ALD_{50} values) were estimated according to the method of Wright(8).

Results. The anti-appetite action of methylphenidate (Ritalin), pipradrol (Meratran), d-desoxyephedrine (methamphetamine) and d-amphetamine was studied first in cats. AAI values obtained with various doses of these compounds are shown in Table I. Food consumption data for all 4 compounds are shown in Fig. 1. It is at once apparent that the piperidine stimulants are, in cats, potent anorexiants. Moreover, the anti-appetite effect is both quantitatively and qualitatively quite similar for piperidine stimulants on one hand and sympathetic amines on the other. In the case of either of the 4 agents listed doses as small as 0.25-0.40 mg/kg delayed the eating response by half an hour or more; however, cats started eating again before the second administration of the compound approximately 7 hours later. With larger doses AEL increased until, with doses of about 1 mg/kg, cats did not touch the food for at least 8 hours so that the compound was given the second or sometimes the third time before the animal started eating. Within the lower dose range (0.25-0.63 mg/kg) the drug effect seemed confined to delaying the first food intake; upon termination of medication food consumption was resumed at normal rate. This is indicated by the fact that the slopes of control and experimental food consumption curves were parallel for these doses (Fig. 1). At higher dose levels not only did the cats refrain from eating during as well as for some time after

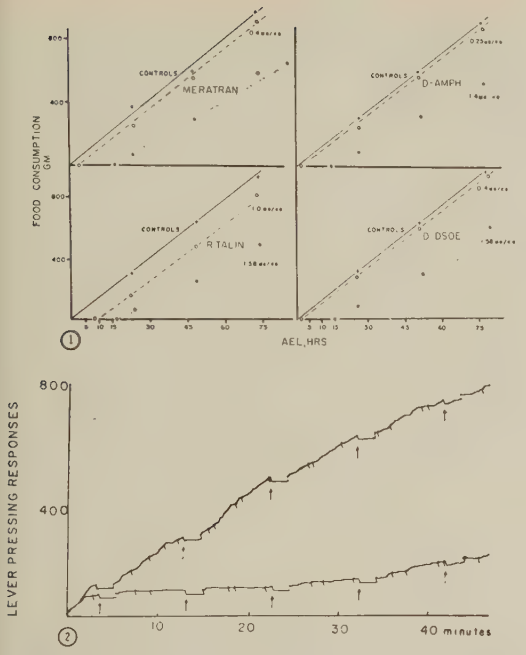


FIG. 1. Anti-appetite effects of d-desoxyephedrine (D-DSOE), d-amphetamine (D-AMPH), pipradrol (Meratran) and methylphenidate (Ritalin). Abscissa: cumulative food consumption in g. Ordinate: Average Eating Latency (AEL) in hr. Figure represents data obtained for 2 dose levels/agent, 5 cats/dose. Controls represent average food consumptions, 15 cats for each curve, recorded during week preceding experiments. Other legends in the figure.

FIG. 2. Effects of pipradrol (Meratran) on conditioned emotional response (CER) of a rat. Upper curve—control CER. Lower curve—CER following pipradrol, 2 mg/kg, s.c. Abscissa—cumulative index of lever pressing; slope of the record indicates rate of lever pressing. Ordinate—time in min. Short strokes —aperiodic positive reinforcement (food pellet). Depression in the record indicated conditioned stimulus in form of a clicking noise introduced at the arrow and terminated 2 minutes later by electric shock.

termination of the drug treatment, but the slope of the curve for experimental food consumption was considerably flatter than that for controls (Fig. 1). This was also reflected by a drastic increase of the anti-appetite index (AAI) at higher dose levels (Table I). At these dose levels average food consumption was lowered from the average control value of about 350 g per day to 175 g or less.

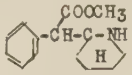
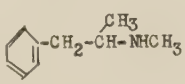
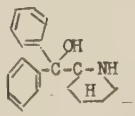
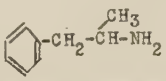
It can be said that at lower dose levels eating latency expresses duration of drug action since, when the latent period is over, normal eating habits are resumed. Contrarily, with larger doses there is a change in rate of food consumption which could last for several days (Fig. 1). That relatively large doses of piperidine and amphetamine-like stimulants seriously impair feeding was borne out also in a separate experiment in which particularly well nourished cats were given single doses of 1.58 mg/kg of methylphenidate, pipradrol, d-amphetamine and methamphetamine, 3 cats per agent, on 4 consecutive days. These cats ate from 75 to 100 g of food per day and lost between 10 and 15% of their body weight during this time period.

Anorexigenic activity of the compounds in question was further tested in rats trained in bar pressing for food reward. Since piperidine stimulants are referred to as ataractics, *i.e.* tension-reducing agents(9) it was of interest to test also whether or not these agents would affect bar pressing in rats with conditioned emotional response (CER) superimposed over their operant behavior. Doses of 1.0 and 2.0 mg/kg of methylphenidate, pipradrol, methamphetamine and d-amphetamine decreased significantly operant behavior in both unconditioned and CER rats (*cf.* Fig. 2 for effect of pipradrol on a CER rat). Similar findings were reported for d-amphetamine by Dews (10) in certain conditions of pigeon operant behavior; to the contrary, Brady(5,11) found with the same compound an increase of operant behavior in CER rats.

To complete the comparison between piperidine excitants and amphetamine-like compounds, the potency of these compounds to stimulate coordinated motor activity was measured in rats, and their toxicity in mice and cats. The results together with the data on cat anorexia are shown in Table II. Some of the experiments on motor activity and toxicity reported here were carried out earlier in separate investigations(1,2,6) dealing with single compounds of the present series; agreement between various sets of data is close. There appears to be little difference between the compounds in question with regard to both their pharmacologic activity and toxicity.

Discussion. This study demonstrates potent anorexiant action in experimental animals of 2 piperidine excitants, methylphenidate (Ritalin) and pipradrol (Meratran). It also provides data for a comparison of this action as exhibited by Ritalin and Meratran on one

TABLE II. Anti-appetite, Motor Stimulant and Toxic Actions of Methylphenidate, d-desoxyphedrine, Pipradrol and d-amphetamine. Chemical structures represented so as to emphasize similarity of compounds in question.

Compound	Structure	Anti-appetite action MED, mg/kg	ALD_{50}, mg/kg cats	Toxicity LD_{50}, mg/kg mice	Motor activity MED, mg/kg
		p.o.			
Methylphenidate HCl (Ritalin)		.33	85	285 ± 25	1.6
Methamphetamine HCl (d-desoxyephedrine)		.33	50	180 ± 35	1.0
Pipradrol HCl (Meratran)		.40	45	168 ± 18	1.5
d-amphetamine SO_4		.25	43	83.5 ± 6	.7

hand and amphetamine and methamphetamine on the other, on the basis of which some suggestions may be made with reference to the mechanism and site of action of the older amphetamine-like agents on which there is no unanimous agreement(3). Meratran and Ritalin are probably devoid of peripheral smooth muscle activity(1,2); their anorexigenic action therefore should be central. The close qualitative and quantitative resemblance between anorexigenic action of piperidine stimulants on one hand and that of the 2 sympathetic amines on the other strengthens the suggestion that amphetamine and methamphetamine are also central anorexiants by virtue of their action on the hypothalamus, either directly or *via* the cortex(3,12,13).

Meratran and Ritalin were not previously reported as anorexiants in experimental animals. However, their anorexigenic action is mentioned in clinical literature(14,15). That this is infrequent may be due to the fact that these agents are usually employed in emotionally unstable patients; since appetite of such patients is notoriously variable, the anorexigenic action of piperidine stimulants may easily escape notice. Although it is not our intention to suggest a resemblance between psychotics and the rats with "conditioned emotional response" which were rendered anorexic in this investigation, our data may suggest that anorexia could be a side action of piperidine stimulants in regressed schizophrenics or senile patients in whom their use is indicated. On the other hand, it may suggest their trial as anorexiants in cases of nonpsychiatric obesity.

Anorexigenic action of Ritalin and Meratran constitutes one more pharmacologic property that these compounds share with amphetamine stimulants, the other being their potent motor stimulant (Table II) and their weak analeptic and cardiovascular actions(1, 2). An additional interesting similarity is that both Ritalin and centrally active sympathetic amines are effective antagonists of reserpine depression(1,16,17). It has been hypothesized by Everett and Toman(18) that desoxyephedrine might have replaced in these experiments norephinephrine or related compounds which are depleted in central neurones following administration of reserpine(19). It may be suggested that all 4 compounds studied at present could act in this fashion since they are structurally related having a secondary nitrogen 2 carbons removed from the phenyl ring (Table II).

Expectation was voiced on several occasions (14,15) that the clinical effects of piperidine excitants should differ from those of the am-

phetamine-like agents. This may have been based on the somewhat subjective impression that piperidine compounds, contrary to amphetamine, do not "irritate" or "aggravate" experimental animals(2). On the other hand, a recent editorial(20) warned against undue optimism in expecting distinct clinical differences between piperidine and amphetamine stimulants. The data adduced here support this view entirely since they clearly indicate that there is little pharmacological basis for differentiating between these two classes of compounds.

Summary. 1. Anti-appetite action of d-amphetamine, d-desoxyephedrine, pipradrol (Meratran) and methylphenidate (Ritalin) was tested in selected trained cats. The agents were given s.c., once prior to, and twice following presentation of food. 2. A delay in eating response of half hour or more appears at dose levels of 0.25 to 0.4 mg/kg with all 4 agents. At doses of 0.63-1.58 mg/kg food consumption was reduced by more than half and eating response delayed 10-30 hours. 3. The compounds inhibited also at 1 to 2 mg/kg dose levels the operant behavior (bar pressing for food reward) of unconditioned and conditioned rats. 4. No qualitative or quantitative differences were found among the 4 agents studied with regard to stimulation of motor activity, toxicity and anorexigenic action. 5. Site of action of the anorexigenic effect of the compounds is discussed and similarity of this and other effects of piperidine stimulants on one hand and of amphetamine excitants is stressed.

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Received May 25, 1959. P.S.E.B.M., 1959, v102.

Induced Immunological Unresponsiveness to Staphylococcus Toxoid in Rabbits.*† (25179)

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Many investigators(1-6) demonstrated, by serological methods, that a state similar or identical to actively acquired tolerance(7) can be induced in different animals using a variety of antigens. To our knowledge no reports have appeared which relate actively acquired tolerance to animal's susceptibility to disease. Since many infectious diseases are known to be transmitted *in utero, in ovo,* or neonatally, it is possible that this state could play an important role in the pathogenesis of these diseases. The work reported herein demonstrated that resistance of rabbits to dermonecrotic toxin is related to their neonatal exposure to staphylococcus toxoid.

Methods. Staphylococcus toxin and toxoid. Staphylococcus aureus Wood 46 was used for production of toxin by the method of Leonard and Holm(8) except that the toxin was produced by growing the organism in an atmosphere of 75% CO_2 and 25% oxygen. Toxin was converted to toxoid by addition of formalin to final concentration of 0.3%. The process of toxin-toxoid conversion was followed by the hemolysin test and by dermonecrotic effects in rabbit skin. *Injection of rabbits with antigen.* Neonatal exposure of rabbits to toxoid was accomplished by injecting half the animals in each litter intraperitoneally with staphylococcus toxoid, on each of the first 5 days of life. The dosage was: 1st day 0.1 ml, 2nd day 0.2 ml, 3rd day 0.3 ml, 4th day 0.4 ml, 5th day 0.5 ml. Hyperimmunization of rabbits at 62 days of age was accomplished by injecting 0.5 ml of toxoid intraperitoneally on alternate days for a total of 5 injections. *Hemolysin inhibition titration.* This titration was done on serum samples obtained on the 61st and 92nd day of life. The unit of hemolysin was established by incubating 1 ml of 2% suspension of rabbit red blood cells with 1 ml of serial 2-fold dilutions of toxin. The tubes were incubated 1 hour at 37°C, then one hour at room temperature. The highest dilution giving complete hemolysis was taken as the unit. Two units of hemolysin were used for hemolysin inhibition test in which 1 ml of a 2% suspension of rabbit cells was incubated with a mixture of 2 full units of hemolysin contained in 0.5 ml, and 0.5 ml of a serial 2-fold dilution of serum. *Dermonecrotic effects of toxin.* On the 92nd day of life 0.1 ml of serial 2-fold dilutions of toxin were injected intradermally into rabbits (undiluted, 1/5, 1/25, 1/125). Four days after injection the diameter of resulting lesions was measured and total area of necrosis was computed from these measurements.

Results. The results of first experiment are compiled in Table I. At 61 days of age, the day before hyperimmunization was begun, no animals in Group A and B showed an antihemolysin titer. This and the fact that animals of Group B were also negative at 92 days of age, would indicate that rabbits injected with staphylococcus toxoid during the first 5 days of life were incapable of producing antihemolysins. On the other hand, results obtained with Group C indicated that the rabbit between 2 and 3 months of age was capable of responding by producing antibodies to staphylococcus toxoid. The most critical comparison is that between Groups A and C. Animals in Group C which received toxoid only after reaching 62 days of age showed a relatively high antihemolysin titer and a small area of necrosis, while those in Group A which had received both neonatal and hyperimmunization injections of toxoid, produced less than 1/5 the antihemolysin, and the necrotic area in these animals was almost twice as large as

* Approved for publication as Scientific Journal Series Paper No. 4086 Minn. Agric. Exp. Sta.

† Work supported in part by grant from U.S.P.H.S.

The authors wish to thank Opal Waltz and Paul Holmberg for expert technical assistance.

TABLE I. Effects in Rabbits of Acquired Tolerance on Antihemolysin Production and Resistance to Staphylococcus Toxin.

Group	No. of rabbits	Neonatal inj. 1-5 days	Hyperimm. inj. 62-70 days	Avg antihemolysin titer 61 days	92 days	Necrosis* in cm², 96 days
A	11	Yes	Yes	Neg	1/14	3.5
B	8	"	No	"	Neg	13.6
C	5	No	Yes	"	1/74	2.1
D	6	"	No	"	Neg	17.6

* Avg area of necrosis.

TABLE II. Effects in Rabbits of Acquired Tolerance on Antihemolysin Production and Resistance to Staphylococcus Toxin.

Group	No. of rabbits	Neonatal inj. 1-5 days	Hyperimm. inj. 62-70 days	Avg antihemolysin titer 61 days	92 days	Necrosis* in cm², 96 days	Dead/Inj.
A	10	Yes	Yes	Neg	1/2.4	61	4/10
B	6	"	No	"	Neg	94	4/6
C	6	No	Yes	"	1/24	32	0/6
D	5	"	No	"	Neg	71	2/5

* Avg area of necrosis.

that in Group C.

The second experiment, Table II, confirms in every respect results presented in Table I. Some animals in this experiment died as a result of utilizing a toxin of greater potency. Although the number of animals was small, there is good correlation between size of necrotic areas produced and survival of the animal.

Exps. 1 and 2 show distinct differences not only in size of necrotic lesions produced but also in the character of the lesions which developed in the respective groups. In Group A, which had received neonatal and hyperimmunization injections, necrotic lesions were large, and the diffuse edge of the lesion although visible was relatively indefinite. Contrariwise, in Group C which had received only hyperimmunization injections, lesions were smaller, discrete and surrounded by vivid erythematous ring. These lesions healed faster than those in Group A.

Exp. 3 (Table III), was designed to determine whether Arthus type of sensitization was responsible for some of the necrotic reaction which occurred upon injection of toxin. The procedure used was the same as that employed previously except that toxoid instead of toxin was used to challenge the rabbits. This experiment confirms the serological results of the first 2 experiments and demonstrates clearly that the Arthus reaction was not a factor in development of necrosis.

Discussion. This work is to our knowledge the first demonstration that the state of acquired tolerance is related to actual resistance to infectious disease. If these findings are confirmed and it is established that acquired tolerance is a general immunological phenomenon, it should enable us better to understand the pathogenesis of several important infectious diseases transmitted *in utero, in ovo* or neonatally.

Although actively acquired tolerance and immunological paralysis(9) may bring about the same result, namely, "block" the ability of

TABLE III. Effects in Rabbits of Acquired Tolerance on Antihemolysin Production and Arthus Response to Staphylococcus Toxoid.

Group	No. of rabbits	Neonatal inj. 1-5 days	Hyperimm. inj. 62-70 days	Avg antihemolysin titer 61 days	92 days	Arthus response 96 days
A	8	Yes	Yes	Neg	1/50	Neg
B	6	"	No	"	Neg	"
C	5	No	Yes	"	1/150	"
D	5	"	No	"	Neg	"

the individual to form detectable antibodies, some differences seem to exist between these 2 phenomena. Relatively large doses of polysaccharide antigens which tend to linger in the system for considerable periods of time(9,10, 11) have usually been used to induce immunologic paralysis, whereas the antigens used thus far to demonstrate actively acquired tolerance were proteins. Where studies have been made, proteins disappear from the system in a relatively short time(12,13,14). In addition, whereas immunological tolerance can be induced at any age, actively acquired tolerance could only be induced in individuals before birth or shortly thereafter. Future studies will probably clarify the relationship of these 2 phenomena.

Actively acquired tolerance seems to bear a superficial resemblance to enhancement of mouse mammary tumor transplants induced by certain tissue factors(15,16,17,18). However, the fact that adult mice have been used in studying cancer enhancement phenomenon would tend to discount such a similarity. Further work needs to be done for an accurate assessment of any similarity.

Summary. 1. Rabbits exposed to staphylococcal toxoid early in life have a reduced capacity to respond to the same toxoid in later life as evidenced by development of low antihemolysin titers. 2. Resistance to dermonecrotic effects of staphylococcal toxin is also reduced by early exposure of rabbits to the toxoid. 3. The Arthus response does not play a role in development of lesions under conditions of these experiments. 4. The possible role of acquired tolerance and similar phenomena in infectious diseases is also discussed.

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Received June 5, 1959. P.S.E.B.M., 1959, v102.

Inhibition of Human Salivary and Prostatic Acid Phosphatase and Yeast Enolase by Low Fluoride Concentrations.* (25180)

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The inhibitory effect, *in vitro,* of fluoride on enzymes including enolase and acid phosphatase from several sources is well known. However, little attention has been paid to quantitation of effects which might be observed in the range of possible physiological concentrations of fluoride. The present investigation was designed to study(1) the characteristics of inhibition of acid phosphatases of saliva and prostatic tissue and of enolase by low

* This study supported by grants from U. S. Public Health Service.

concentrations of fluoride and (2) the possibility that a simple micro analytical method for determination of fluoride might be developed by application of experimental technics involved.

Materials and methods. Fluoride inhibition of acid phosphatase of "unstimulated" saliva samples was investigated both by means of a modification of the procedure of Huggins and Talalay(1) employing phenolphthalein phosphate substrate and that of King-Armstrong (2) using disodium phenyl phosphate substrate. Sodium fluoride was added to the buffer solution in a concentration such that the reaction mixture contained the desired concentration of fluoride ion. Samples utilizing phenolphthalein phosphate as the substrate were incubated for one hour at 37°C. Color intensity of samples containing "0" fluoride concentration was called 100% enzyme activity. Prostatic acid phosphatase solutions were prepared according to the procedure of Abul-Fadl and King(3). Analytical procedures were the same as those for saliva except that due to high concentration of acid phosphatase in extracts of the gland, considerable dilution of enzyme preparations was necessary. The stock solution of enzyme was stored frozen and no change in activity over a 3 month period was noted. The effect of fluoride on crystalline yeast enolase was studied by the method of Warburg and Christian(4) which utilizes the change in optical tranmission at 240 mμ during conversion of β-glycerol phosphate to phosphoenolpyruvic acid by enolase as indicator of enzyme activity.

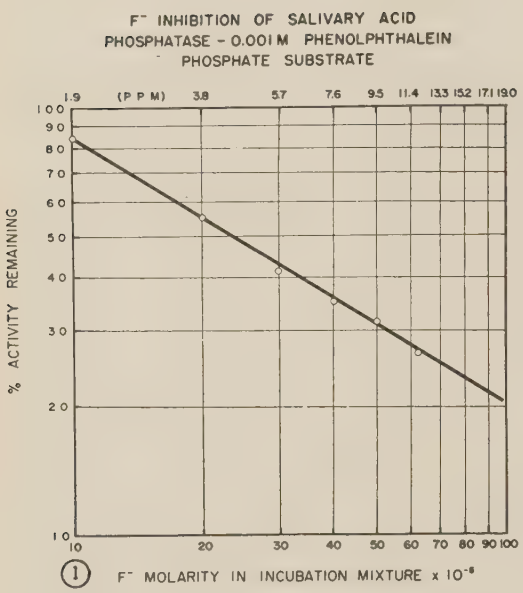
Results. The greatest inhibitory effect/unit of fluoride on salivary acid phosphatase activity occurred over a relatively narrow range of fluoride concentrations and was thoroughly investigated. The results of a typical experiment are illustrated in Fig. 1. When the logarithms of fluoride molarities were plotted on the abscissa and logarithms of activities remaining on the ordinate, a straight line relationship for fluoride concentrations of 10 to 62.5 x 10^{-5} M was obtained. Higher fluoride concentrations gave further reductions in enzyme activity, but the linear relationship described above was no longer followed.

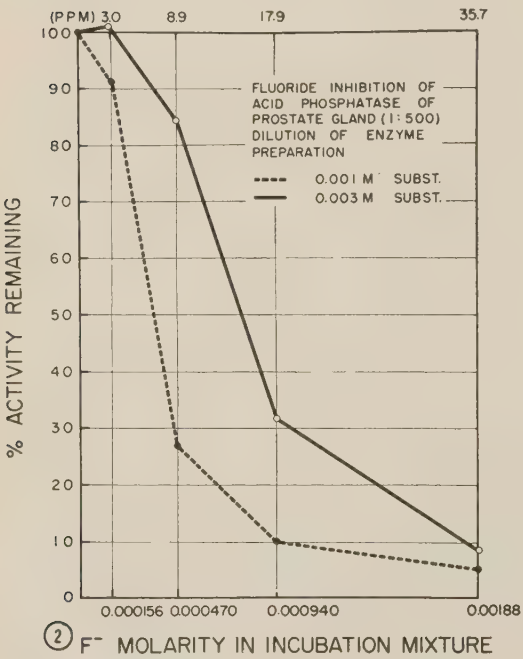
A phenomenon observed with many salivary samples was a slight activation of acid phosphatase activity in the presence of the smaller concentrations of fluoride investigated. For example, in the presence of 7.8 x 10^{-5} M fluoride one sample gave 2% more activity than when no fluoride was added. This observation, and similar data from other samples, was not due to an analytical error, but was observed upon repeated analysis of the same sample. This finding may have been due to a stabilizing effect of small quantities of fluoride on the enzyme rather than an activation. Stabilizing effects of ions against denaturation by factors including heat are known, such as protection of activity of trypsin by calcium(5).

Results obtained by varying substrate concentration of the incubation mixture while keeping the enzyme concentration and fluoride molarity constant, indicated that it was possible to overcome partially the inhibitory effect of the fluoride by increasing the substrate molarity. These experiments were performed with various concentrations of fluoride between 2.0 and 6.25 x 10^{-4} M.

The King-Armstrong phosphatase method using disodium phenyl phosphate as substrate gave similar results. The principal difference between data obtained by this procedure and those of the Huggins and Talalay method was that a higher concentration of fluoride was required to cause inhibition. For example, when disodium phenyl phosphate was the substrate, the acid phosphatase activity was decreased by 53% in presence of 5.0 x 10^{-3} M fluoride. The activity of the same sample was decreased by 61% in the presence of 2.35 x 10^{-4} M fluoride when the substrate was phenolphthalein phosphate. The difference in degree of inhibition of activity was probably due to higher concentration of disodium phenyl phosphate (0.005 M) than that of phenolphthalein phosphate (0.001 M).

Investigations with human prostatic acid phosphatase were largely directed towards use of this enzyme as a simple analytical method for determination of fluoride. This enzyme source was selected because it furnished a stable source of a large quantity of homogenous activity. Whereas 100 ml of saliva





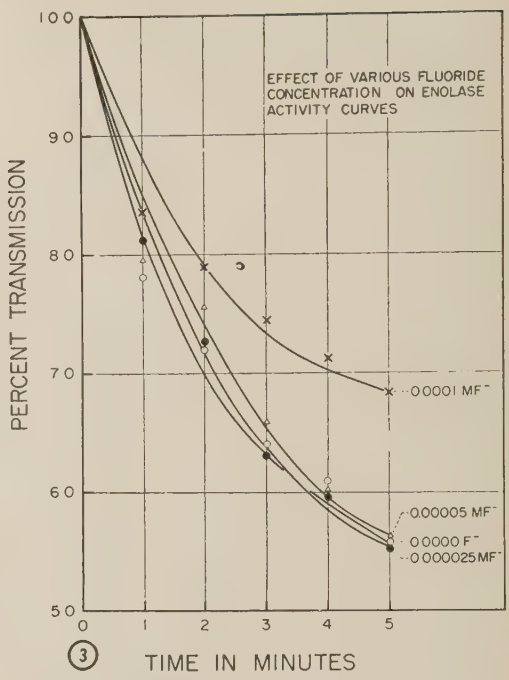


FIG. 1. F⁻ inhibition of salivary acid phosphatase —0.001 M phenolphthalein phosphate substrate.

FIG. 2. Fluoride inhibition of acid phosphatase of prostate gland (1:500) dilution of enzyme preparation.

FIG. 3. Effect of various fluoride concentration of enolase activity curves.

would liberate 5-10 mg of phenolphthalein/hour at 37°C from 0.001 M phenolphthalein phosphate solution, one g of wet prostate tissue released about 1000 mg under the same conditions. All findings with prostatic acid phosphatase discussed subsequently are from experiments in which phenolphthalein phosphate was the substrate.

Experiments with varying enzyme concentrations (0.0001 to 0.0004 g of wet prostate gland/ml of enzyme solution) were conducted to study inhibition of acid phosphatase activity when substrate concentration and fluoride concentrations were constant. The *percent* inhibition was the same for each enzyme concentration tested, although total amount of substance acted upon varied considerably. In Fig. 2 typical data obtained for enzyme inhibition from 2 concentrations of substrate when enzyme concentration was constant, are presented. These data clearly indicate that inhibition of acid phosphatase from the prostate gland by fluoride may also be partially overcome by increasing the substrate concentration. The activating effect of small concentrations of fluoride under certain conditions is again illustrated.

The range of greatest inhibitory effect/unit of fluoride on activity of acid phosphatase of

the prostate gland was thoroughly investigated. When the data were plotted on log-log scale in the same manner as results of salivary acid phosphatase studies, a straight line relationship similar to that of Fig. 1 was obtained. Application of the straight line relationship to possible analysis of fluoride was considered. The percent inhibition of the acid phosphatase from a single prostate gland was constant with a given fluoride concentration. Thus once the percent inhibition of activity by various fluoride concentrations was well established, it was possible to determine the fluoride content of pure aqueous solutions. A 3 ppm solution of fluoride analyzed in duplicate tubes on 6 different days gave 8-11% inhibition of activity. Results of analysis based on a single assay in duplicate were 3 $\pm$ 0.4 ppm of fluoride. The error of determination could readily be reduced by using a series of replicate tubes for a single assay. Higher concentrations of fluoride gave a smaller analytical error on a percentage basis. Attempts were made to apply this procedure to direct analysis of fluoride in blood serum. This method suffered from the disadvantage that proteins in serum stabilized the enzyme preparation and resulted in elevated activities. If the method is applied to tissue samples, separation of proteins and fluoride must be accomplished in a preliminary procedure.

In Fig. 3, curves show effects of varying concentrations of fluoride on activity of yeast enolase as measured by conversion of beta glycerol phosphate to phosphoenolpyruvic acid. The "0", 0.5, and 1 parts/million fluoride curves are essentially the same and indicate that at these concentrations fluoride did not appear to greatly influence conversion of substrate by the enzyme. At 1.96 ppm there is marked inhibition of enzymatic activity. This is reflected by 30% reduction in amount of substrate acted on in 5 minutes.

The concentration of fluoride (2-3 ppm) required to cause significant inhibition was much above the 0.2 ppm found in body fluids (6). It is inconceivable in view of the rapid excretion of fluoride, the sequestration of fluoride by calcified tissues, and the total volume of body water, that concentrations of fluoride would reach a level which would inhibit the activity of these enzymes, with the possible exception of a transitory effect on salivary acid phosphatase, by addition of 1.0 to 1.2 ppm of fluoride to drinking water.

Summary. A study was made of the effects of low concentrations of fluoride on activity of salivary and human prostate acid phosphatase and yeast enolase. Concentrations of fluoride or concentrations that could be derived from fluoridation of drinking water did not significantly alter enzymatic activity. The straight line relationship between the logarithms of low fluoride concentrations and logarithms of percent of enzyme activity remaining suggested that it is possible to analyze fluoride content of pure aqueous solutions by degree of inhibition of activity of acid phosphatase of human prostate tissue.

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Received June 15, 1959. P.S.E.B.M., 1959, v102.

Studies on Mammary Tumor Inducing Virus in Mice (Bittner Virus).* (25181)

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Biological, biophysical and biochemical methods have been employed in studies on properties of mammary tumor inducing Bittner virus(1-3). These studies demonstrated non-lipid fraction of fresh milk of agent-harboring mice to be best source of virus, and enzymes, such as trypsin, chymotrypsin, prostate phosphatase, and snake venom diesterase, and chelating agents, have little influence on virus present in milk(4). Electron microscope studies of sections of mammary tumors from virus-carrying mice revealed virus-like particles in these tumors(5-6), but not in lactating breast tissue of virus-carrying mice(4-7), known to be good source of virus(8). Attempts to correlate electron microscope observations with bioassays of these tumors for tumor-inducing activity failed to produce conclusive evidence that the observed particles are the Bittner virus(9). Experiments were therefore undertaken to study the morphological, biophysical and biochemical properties of the agent present in milk of mice.

Material and methods. Milk from mice of virus-carrying A, genetically identical virus-free Af, and RIII virus-harboring strains was used as source of the agent. Bioassays were carried out in 1 to 4-week-old $(C_{57}$ x Af$)F_1$ female mice to determine tumor-inducing activity following each treatment of milk. For electrophoretic fractionation, milk was defatted by centrifugation at 600 x g for 20 minutes, the remaining fraction decaseinated with chymotrypsin (0.2 mg/ml) for 5 minutes at 38°C. Coagulated milk was centrifuged at 600 x g for 20 minutes and supernatant at 105,000 x g for 2 hours at 4°C. Part of resulting pellet resuspended in citrate buffer of ionic strength 0.01 at pH 6.0 to the original volume of milk sample was used for bioassays, the rest for zone electrophoresis at 4°C in a trough with powdered cellulose in citrate buffer with current of 450 V and 15 m.a. After 20 hours, cellulose block was cut in ½ inch segments, buffer removed from each segment by filtration through separate filters with Whatman No. 1 paper and analyzed for phosphorus(10) and protein(11). High-speed centrifugal pellets of defatted and decaseinated milk were tested for presence of RNA and DNA. Ribonucleic acid was determined in hot perchloric acid extracts of pellets by modified orcinol method(12) and by ultraviolet absorption readings in Beckman DK2 recording spectrophotometer. For DNA determinations, Burton-Dische reaction(13) was used directly on the pellet. Ribonuclease digestion of pellet material was carried out at 38°C for 30 minutes with 10 μg of ribonuclease/ml of pellet resuspended in physiological saline, citrate buffer or distilled water. Material treated was then centrifuged at 105,000 x g for 2 hours at 4°C, and the supernatant and sediment tested for acid-soluble and insoluble RNA and tumor-inducing activity. High-speed centrifugal pellet material was treated with 75% cold ethanol, precipitate spun at 600 x g for 20 minutes at 4°C, resuspended to the original volume of defatted, decaseinated milk and tested for tumor-inducing activity. Phenolic deproteinization of defatted milk or of high-speed centrifugal pellets from such milk was carried out according to Gierer and Schramm technic(14), modified by Kirby (15). Biphthalate or salicylate buffer (0.15 M pH 7.3) was used as diluent. Fluorocarbon deproteinization technic(16-19), using 1, 1,2-trichloro-1, 2,2-trifluoroethane (Genetron 113), was also employed in attempt at extraction of the virus from high-speed centrifugal pellets of defatted and decaseinated milk.

* This work supported by Research Grants from Nat. Cancer Inst., N.I.H., U.S.P.H.S., and Am. Cancer Soc.

TABLE I. Bioassays of Various Fractions of Virus-Carrying Strain A Milk in (C_{57} × Af)F_1 Mice.

Numerators = Mice with mammary tumors. Denominators = Mice alive at earliest tumor appearance. Duration of bioassays—18 mo.

Type of fraction tested (treatment)	Dilutions 10^{-2}	10^{-3}	10^{-4}
Defatted milk (600 × g for 20 min.)	16/20 80% 12M*	12/16 75% 12M	13/18 72% 10M
Whey (incubation with chymotrypsin)	7/19 37% 17M	12/20 60% 12M	3/15 20% 16M
Supernate (10^{-1}) (105,000 × g for 2 hr)	12/19 63% 12M		
Pellet (105,000 × g for 2 hr)	9/20 45% 11M	10/19 53% 14M	11/22 50% 10M

* Avg tumor appearance in mo.

Electron microscope studies of ultrathin sections of pellets of milk from RIII, A, and Af strain mice were carried out according to method previously described (20).

Results. Bioassays of fractions of A strain milk following different treatment, preceding electrophoretic fractionation, are shown in Table I. High-speed centrifugal pellet from defatted, decaseinated milk retained tumor-inducing activity. Activity of supernatant may be due to resuspension of some of particulate material before or during removal of the supernatant. Chemical analysis for phosphorus and protein of high-speed centrifugal pellet (Table I) from defatted and decaseinated strain A virus-carrying milk after zone electrophoresis is shown in Fig. 1. On the basis of Folin-Lowry test, all segments of the cellulose block were pooled into 3 fractions for bioassays. Most of protein and 65% of phosphorus was found in Fraction II or central segment around the origin, and only small quantities in Fractions I and III towards anode and cathode. Bioassays of electrophoretic fractions of high-speed centrifugal Strain A milk pellet (Table II) showed tumor-inducing activity only in Fraction II. The tumor-inducing particle, therefore, does not appear to have a large net charge at pH 6. Electrophoretic analysis of strain Af milk with no tumor-inducing activity gave a pattern essentially similar to that of strain A milk with high tumor-inducing activity.

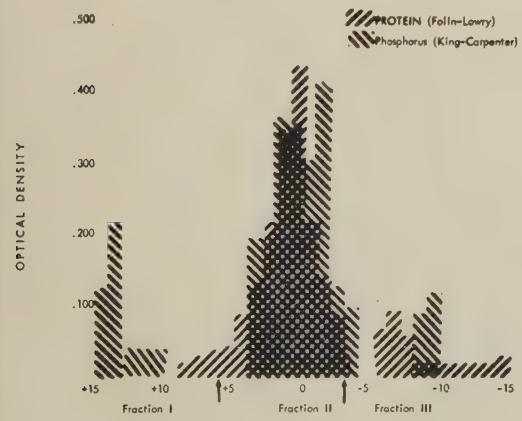

FIG. 1. Electrophoretic pattern of high-speed centrifugal pellet from defatted, decaseinated, virus-carrying strain A milk.

The amount of RNA in centrifugal pellets of defatted and decaseinated strain A virus-containing milk ranged in samples tested from 8 to 23 μg/ml of original milk. Estimations of RNA content in pellets of similarly treated virus-free Af strain milk have so far been unsuccessful because of color interference with the orcinol reaction. Similarly prepared pellets from virus-harboring A and virus-free Af milk showed no detectable DNA by method which could have shown quantities greater than 0.2 μg/ml of original milk. Absorption spectra of extracts of pellet material from both types of milk were characteristic of nucleic acids and agreed quantitatively with results of orcinol reaction. Ribonucleic acid analysis of high-speed centrifugal pellet from strain A milk before and after ribonuclease treatment (Table III) demonstrated alteration in solubility and sedimentability of RNA in the original pellet. Small fraction of RNA appeared to be resistant to ribonuclease. Bioassays on ribonuclease-treated high-speed cen-

TABLE II. Bioassays of Electrophoretic Fractions from High-Speed Centrifugal Pellet (105,000 × g—2 Hr) of Strain A Milk in (C_{57} × Af)F_1 Mice.

Numerators = Mice with mammary tumors. Denominators = Mice alive at earliest tumor appearance. Duration of bioassays—18 mo.

Type of fraction tested		Dilutions 10^{-2}	10^{-3}	10^{-4}
I	Anode side	0/19	0/18	0/19
II	Origin area	20/20 100% 13M	8/17 47% 12M	1/18 6% 18M
III	Cathode side	0/15	0/20	0/20

trifugal pellets from defatted or defatted and decaseinated strain A milk (Table IV), although not complete, show at least some tumor-inducing activity remains following this, and after cold ethanol treatment.

TABLE III. Ribonucleic Acid Analyses of High-Speed Centrifugal Pellet (105,000 × g—2 Hr) of Strain A Milk before and after Ribonuclease Treatment.

Material (fraction)	Amt of ribonucleic acid/10 ml Acid soluble	Acid insoluble	Total (μg)
Original pellet (I)	103	131	234
Pellet after ribonuclease treatment (II)	20	10	30
Supernate from (II) (105,000 × g)	25	154	179

Aqueous phase from phenolic deproteinization of strain A milk has, so far, shown no tumor-inducing activity (after 14 months of bioassay duration) and control material considerable activity (86% tumor incidence in 9 months). The aqueous layers contain nitrogen in quantities of 80-140 μg/ml of original milk. This represents 1-2% nitrogen of original defatted milk. Bioassays of aqueous phase from fluorocarbon deproteinization of milk, because of short duration, do not permit any conclusion.

High-speed centrifugal pellets of virus-harboring RIII and A strain and of Af virus-

TABLE IV. Bioassays of High-Speed Centrifugal Pellets of Strain A Milk following Ribonuclease or 75% Ethanol Treatment in (C_{57} × Af)F_1 Mice.

Numerators = Mice with mammary tumors. Denominators = Mice alive at earliest tumor appearance.

Duration of exp. (mo)	Material tested	Dilutions 3.2×10^{-1}	2×10^{-1}	10^{-1}	10^{-3}
11	Milk defatted & decaseinated			2/15 13% 10M	
	Pellet incubated		6/23 27% 9M		
	Pellet treated with ribonuclease		3/22 14% 6M		
	Pellet treated with ethanol	5/25 20% 10M			
10	Milk defatted & decaseinated			0/27	
	Pellet treated with ribonuclease			9/42 21% 7M	
9	Milk defatted				1/16 6% 9M
	Pellet untreated				3/17 18% 7M
	Pellet treated with ribonuclease			2/19 11% 6M	2/17 12% 6M
	Supernate from treated pellet			2/19 11% 6M	0/19

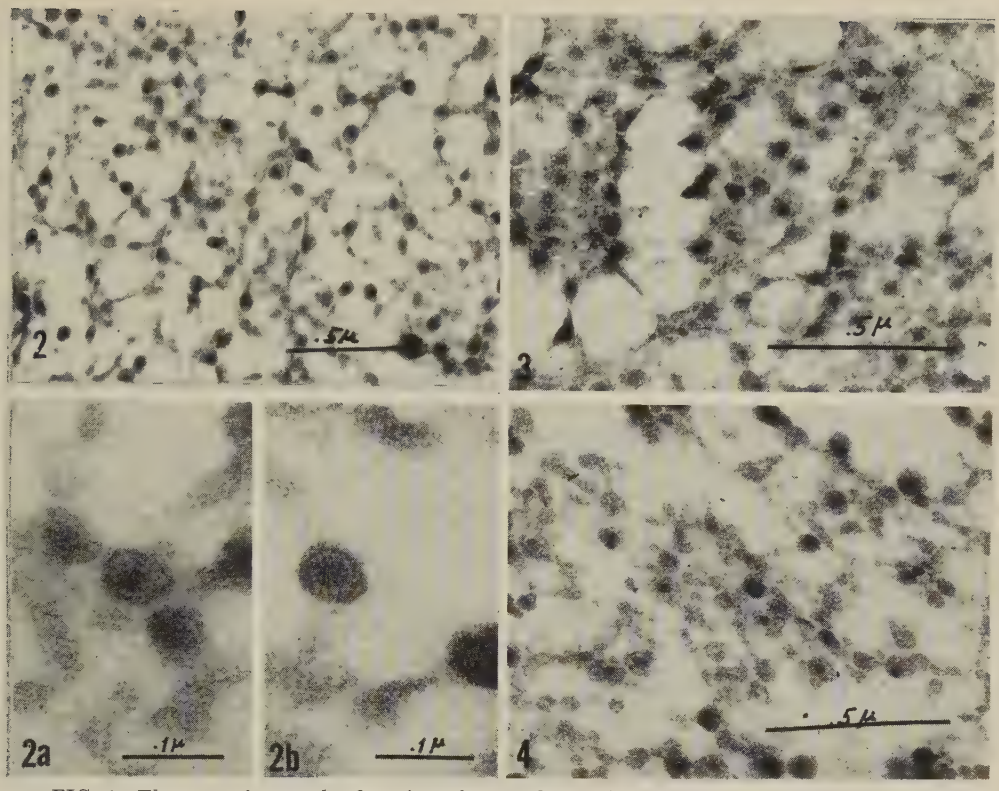

FIG. 2. Electron micrograph of section of upper layer of high-speed (105,000 × g—2 hr) centrifugal pellet from defatted, decaseinated virus-carrying RIII strain milk. Particles with characteristic structure. × 30,000.

FIG. 2a & 2b. Selected fields of Fig. 2 at higher magnification. × 130,000.

FIG. 3. Electron micrograph of section of upper layer of high-speed centrifugal pellet from defatted, decaseinated virus-carrying A strain milk.

FIG. 4. Selected field of upper layer of high-speed centrifugal pellet from similarly treated virus-free Af strain milk. × 50,000.

free strain defatted and decaseinated milk have similar macroscopic appearance. They are composed of an upper pale layer and lower more dense brownish zone. Electron microscope studies of sections of upper layer of pellets have revealed characteristic particles of 700 Å average diameter, composed of inner dense core, and outer pale zone, surrounded by 2 limiting membranes. They have been found in every section of upper layer of high-speed centrifugal pellets from RIII and A strain milk (Fig. 2, 2a, 2b, and 3). Similar particles have only occasionally been found in sections of upper layer of pellets from Af strain milk (Fig. 4). Similar study of lower zone of pellets from milk of all 3 strains of mice has shown only amorphous material. High-speed centrifugal pellets from aqueous phase of fluorocarbon-treated pellet material from milk of the 3 strains have macroscopic appearance similar to that of pellets before treatment but contain a much smaller lower layer. In the electron microscope apparently unaltered particles are present in the upper layer (Fig. 5). Denatured protein at the interphase between the aqueous and fluorocarbon layers has failed to reveal any characteristic particles.

Discussion. Bioassays of different fractions from virus-containing strain A milk, following defatting, decaseination, high-speed centrifugation, have demonstrated high-speed centrifugal pellets of treated milk to be suitable starting material for biophysical and biochemical study of the agent. Electrophoretic fractionation has led to localization of tumor-inducing activity in the electrophoretic pattern. Contamination of other fragments has not been observed in the present electrophore-

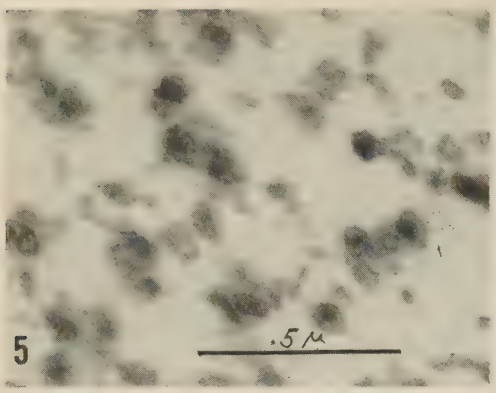

FIG. 5. Electron micrograph of section of upper layer of high-speed (105,000 × g—2 hr) centrifugal pellet from aqueous layer of Genetron-treated high-speed centrifugal pellet from defatted, decaseinated RIII strain milk. × 52,000.

sis experiments. Previous attempts by column electrophoresis showed tumor-inducing activity in varying amounts in all fractions(4), probably from cross-contamination during elution. The present studies indicate more extensive electrophoresis studies of the virus are feasible.

Studies on presence of RNA and DNA in high-speed centrifugal pellets from virus-carrying, defatted and decaseinated A strain milk were possible because of removal of a substance (possibly lactose) which interfered with previous attempts at colorimetric determination of RNA in mouse milk. It should be pointed out that the interfering substance is not always removed by high-speed centrifugation. For this reason, RNA determinations in pellets of similarly treated Af strain milk have so far not been successful. The absence of DNA in pellets of virus-carrying milk, at least in quantities greater than 0.2 μg/ml, is of interest. It should be pointed out that Bittner virus is of cytoplasmic origin, as shown in fractionation studies of tumor cells by differential centrifugation(21) and as indicated by electron microscopy(4-7,22-23). Presence of tumor-inducing activity following ribonuclease treatment of high-speed centrifugal pellets from defatted and decaseinated milk, and treatment with cold ethanol may prove helpful in isolation of the virus from mouse milk.

Attempts to demonstrate virus-like particles in lactating breast tissue of virus-carrying mice similar to those in cytoplasm of mammary tumor cells have been unsuccessful(4-7). Observation of characteristic particles in high-speed centrifugal pellets from virus-carrying milk which have shown considerable tumor-inducing activity is therefore of interest. However, the particles are smaller (700 Å) than those in tumor cells (900 Å). It is not known whether the difference in size, assuming the particles are the same, is the result of treatment of milk and subsequent preparation of pellet material for electron microscopy. Fluorocarbon deproteinization of pellets from virus-carrying milk does not appear to affect the appearance of particles to any appreciable extent, although they decrease in number. Presence of a small number of similar particles in pellets from Af milk, with no tumor-inducing activity, requires exploration. Particles of similar size and appearance to those in virus-carrying mammary tumors have also been observed in some tumors from agent-free mice(7,9). The incidence of mammary tumors in strain Af mice of our colony is about 3% at an age between 400-600 days. In the dairy colony of this strain, only one mammary tumor has, so far, been observed. It is not known whether the smaller number of particles observed in Af milk compared with that in RIII and A strain milk is connected with difference in tumor incidence of these strains (70% at 389 days average age in RIII and 66% at 382 days in A strain).

Further studies based on biochemical fractionation combined with electron microscope examination of sections and bioassays of various milk fractions may finally demonstrate the relationship of these and other particles (24-25) to the tumor-inducing activity of mouse milk. It has been pointed out(4) that shadow-cast preparations of mouse milk do not differentiate virus from calcium caseinate and lipoprotein particles present in milk.

Summary. 1). Zone electrophoresis of high-speed centrifugal pellets from virus-containing defatted and decaseinated strain A milk has led to good recovery of mammary tumor-inducing activity and to its localization in electrophoretic pattern. Nucleic acid determinations have shown presence of considerable quantities of RNA in pellets from similarly

treated A strain milk and absence of DNA. High-speed centrifugal pellets from defatted and decaseinated strain A milk treated with either ribonuclease or cold 75% ethanol retained tumor-inducing activity. 2) Characteristic particles of 700 Å average diameter, have been observed in sections of high-speed centrifugal pellets of agent-harboring RIII and A strain milk which have shown high tumor-inducing activity. They have also been found following treatment of pellets with fluorocarbon. Similar particles have been observed in considerably smaller number in pellets of Af strain milk without tumor-inducing activity. The relationship of these particles to the origin of mammary tumors of mice requires further study.

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Received June 15, 1959. P.S.E.B.M., 1959, v102.

Use of Contrasting Fluorescent Dye as Counterstain in Fixed Tissue Preparations. (25182)

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One of the major problems in fluorescent antibody studies is the nonspecific uptake of fluorescein dye in formalin fixed tissues which often makes differentiation between specific and nonspecific fluorescence difficult. It was observed that a conjugated bovine antiserum using Lissamine rhodamine RB 200 (LR), according to Chadwick *et al.*(1), not only stained the specific organism but imparted a reddish-orange background to the tissue as well. An investigation was made as to the possibility of using LR-conjugated normal serum as a nonspecific counterstain to eliminate the nonspecific fluorescence of the fluorescein dye.

Materials and methods. Normal bovine, ovine and rabbit whole sera and bovine serum albumin were conjugated with Lissamine rhodamine RB 200* according to Chadwick

* Lissamine rhodamine RB 200 was kindly supplied by Arnold, Hoffman, and Co., Providence, R. I.

et al.(2). For proper conjugation it was necessary to add 0.5% phenol to the serum. One g of LR and 2 g of phosphorus pentachloride (stored over P_2O_5) were ground in a mortar for 5 minutes. Ten ml of acetone (dried over $CaCl_2$) were added and allowed to stand 10 minutes with occasional stirring. The mixture was then filtered. To each 2 ml of diluted serum (equal volumes of serum and 1% phenolized normal saline stored at 4°C for 5 days), 1 ml of carbonate-bicarbonate buffer, pH 9, was added. The mixture was then cooled (0-2°C). With slow stirring, to prevent frothing, 0.1 ml of the acetone filtrate/2 ml of diluted serum was added for 15-20 minutes, care being taken to keep the mixture basic. Stirring was continued overnight at 4°C. The mixture was dialyzed against phosphate buffered saline (pH 7.2) until excess dye was removed. Antisera against bacterial, viral and mycotic agents and goat anti-rabbit were conjugated with fluorescein isothiocyanate according to Marshall *et al.*(3). One volume of LR-conjugated serum was added to 20 volumes of the fluorescein-labeled serum. This was applied to clinical necropsy material from humans and swine as well as material from experimentally infected mice, rats, rabbits and pigeons. The material consisted of impression smears, frozen and formalin fixed paraffin embedded tissue. Also 4 primary and 4 continuous cell culture lines infected with viral or bacterial agents were used. Goat anti-rabbit was applied using the indirect technic. Smears, frozen sections and tissue culture preparations were fixed in 10% neutral formalin for 10-15 minutes, then washed 2 times in buffered saline for 10 minutes. In all preparations, normal controls were used. Stained smears were washed in phosphate-buffered saline, pH 7.2, and mounted in glycerol, pH 7.4. A Zeiss fluorescent microscope with OSRAM HBO 200 light source was used, with either a BG12 exciter and GG4-OG4 barrier filter, or a UG2 exciter and GG4 barrier filter.

Results. The LR was nonspecifically taken up where there was no antigen-antibody reaction. Specific staining by the fluorescein-conjugated antiserum was in no instance prevented or overshadowed in the 14 bacterial, 3 viral, and 4 mycotic systems evaluated. In effect, the specific staining of the fluorescent labeled sera appeared enhanced against a contrasting reddish-orange background. Bacteria in tissues and tissue culture as well as in dried smears took up the LR nonspecifically when not of the homologous species.

Discussion. The problem of nonspecific uptake of fluorescein has plagued many workers. Heretofore it has been necessary to precipitate the globulin fraction and to absorb the conjugated antisera to reduce this phenomenon in tissues and tissue culture, which resulted all too often in lowering the serum titer as well as the volume of the final product. In our experience it was generally unnecessary to precipitate or absorb the sera when using this counterstaining technic. Hughes (4) observed that in the differentiation of neoplastic and non-neoplastic tissue, similar results could be obtained with fluorescein conjugated normal rabbit globulin as with fluorescein labeled protein complexes and that any interpretation of results obtained by fluorescent technics would require caution and careful control staining when evaluating changes attributed to an antigen-antibody reaction. King *et al.*(5) found this to be true. Using normal globulin from 14 species of animals and man, he found results so similar as to be indistinguishable in degree and kind from those observed with rabbit sera. Globulins stained normal tissue but not malignant tissue. Louis(6) confirmed that fluorescein-serum protein may be used as a serologically nonspecific stain for normal tissue and attributed the reaction to presence in the normal cell of a protein complex whose isoelectric point is sufficiently different from that of the serum proteins to allow protein-protein interaction. Therefore as the LR-labeled normal sera react under these circumstances, it is presumed to be a protein-protein physico-chemical interaction, depending on factors other than serological. This system now makes it possible not only to better visualize the sites of antigen deposits but also to detect smaller bacteria and viral aggregates in formalin fixed tissues and tissue cultures.

Summary. A counterstaining method is described that gives a contrasting reddish-orange background when used with fluorescein-labeled antibody systems. It curtails non-

specific fluorescence in tissues and tissue cultures. The possibility of a nonspecific protein-protein reaction is discussed. This reaction apparently plays no part in the serological system to which it has been added.

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Received June 18, 1959. P.S.E.B.M., 1959, v102.

Characteristics of Growth of Encephalomyocarditis Virus in Cultures of Mouse Muscle.* (25183)

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Encephalomyocarditis virus (EMC) has been cultivated in embryonic mouse tissues by several investigators. Sanders and Jungeblut (1) cultured the Columbia SK strain through 200 consecutive passages in suspended embryonic mouse brain. Both the Columbia SK and MM agents have been propagated in suspensions of embryonic mouse brain, lung, small intestine, cardiac muscle, and tail and in minced chicken embryo by Shean and Schultz(2); no evidence of multiplication was detected when tongue, liver, kidney, spleen or skeletal muscle of embryos were used. Similar observations have been reported by Chambers, Smith, and Evans in tissue cultures of mouse embryo, embryonic intestine, and urinary bladder of hamsters(3). Growth did not occur in mouse intestine or in hamster subcutaneous tissue and skeletal muscle in plasma clot preparations. The encephalomyocarditis virus has also been grown in non-embryonic tissues. Chambers, Smith and Evans(4) recorded its cultivation in suspended human or mouse testicle. Smith and Evans(5) noted that it grew rapidly and produced incomplete degeneration of roller-tube cultures of monkey testicular tissue. Similar findings were reported by Fabiyi(6) using mouse testes as source of cells. Although Mengovirus multiplies in and disrupts Ehrlich ascites tumor cells *in vivo*(7), no evidence of a cytopathogenic effect could be demonstrated *in vitro* by Flanagan and Colter(8). On the other hand, frequent oncolysis of these tumor cells by the encephalomyocarditis virus was observed by Levy and Snellbacker(9). Multiplication of this virus with development of a cytopathic effect in cultures of KB (human epidermoid carcinoma), L, and HeLa cells has been described by Eagle, *et al.*(10), and Jungeblut and Kodza(11): the latter were unable, however, to cultivate the Columbia SK and MM strains on KB cells and the F virus on any but L cells. Our purpose is to report the successful propagation of encephalomyocarditis virus in cultures of abdominal wall muscle of mice and to describe the characteristics of regularly observed cytopathic changes.

Methods. The strain of encephalomyocarditis virus used, was obtained from Dr. Monroe Eaton, Harvard Medical School. It was passed by repeated intraperitoneal injection into mice and a pool of infected brain prepared and stored at –20°C. The muscle for making tissue cultures was obtained from *adult* mice killed by ether. Immediately after death, the skin was reflected and the entire mass of anterior abdominal musculature removed aseptically. After washing twice in Hanks' solution containing 100 units of penicillin and 50 μg of streptomycin, the tissue was minced with sharp scissor until all frag-

* This work was supported by grant from Riker Labs., Los Angeles, Calif.

ments were 1 mm or less in diameter, then washed 3 times in Hanks' solution and suspended in a small quantity of this fluid. One drop of this suspension containing 10 to 20 tissue fragments was embedded in 0.1 ml of fresh heparinized chicken plasma on the wall of Pyrex tubes (15 x 150 mm) and a drop of chicken embryo extract added to clot the plasma. Two ml of medium (10 ml of inactivated horse serum, 10 ml of beef embryo extract, 40 ml of calf amniotic fluid, 40 ml of Hanks' solution, 50 μg of streptomycin and 100 units of penicillin/ml, and 0.5 ml of 1% soybean antitrypsin) were added and the cultures incubated at 37°C in stationary horizontal position for 6 to 7 days. Most tissue fragments showed considerable outgrowth at this time; the medium was changed and 0.2 ml of 10^{-7} dilution of encephalomyocarditis virus-infected mouse brain pool ($LD_{50} = 10^{-8.3}$) inoculated into each tube. The cultures were then incubated in horizontal position at 37°C and examined daily for cellular changes. To determine the curve of viral growth, aliquots of fluid were removed from cultures at 10 minutes, 24, 48, 72, 96, and 120 hours; the material from several tubes was pooled and titrated for virus content by intraperitoneal injection in mice. To establish the fact that the observed cytopathic effect was due to encephalomyocarditis virus, studies using specific immune serum prepared in rabbits by intraperitoneal injection of mouse brain pool containing the virus were carried out. The serum was diluted 4-fold serially and an equal volume of fluid containing 200 CP_{50} of 6th passage virus added to each serum dilution. After mixing and incubating at 37°C for 2 hours, the serum-virus mixtures were inoculated intraperitoneally into mice and into tissue cultures of mouse muscle. Controls consisted of virus alone, normal rabbit serum mixed with virus, and rabbit immune serum alone. Cultures were observed for development of cytopathic changes and the mice for paralysis or death, for 2 weeks.

Results. Cultures of mouse muscle inoculated with encephalomyocarditis virus infected mouse brain pool revealed a clear-cut cytopathic effect after 48 hours incubation at 37°C. These changes were characterized by extensive destruction of the cellular outgrowth from tissue fragments. Normal cells were replaced by rounded ones which exhibited cytoplasmic granulations and nuclear condensation. The cultures were completely destroyed in about 72 hours. Figs. 1 (prior to inoculation of virus) and 2 (48 hours after infection)

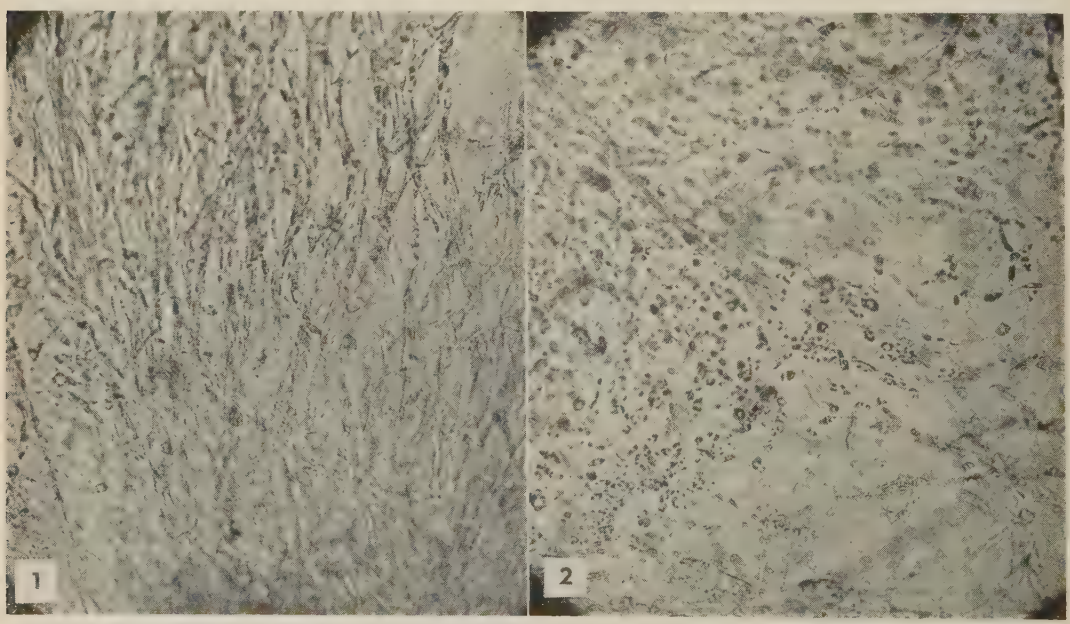

FIG. 1. Appearance of uninoculated mouse muscle tissue culture.
FIG. 2. Appearance of tissue culture 48 hr after infection with EMC virus.

TABLE I. Neutralization of Encephalomyocarditis Virus in Tissue Culture by Specific Immune Serum.

Serum dilution	Positive cytopathic effect*	Death in mice
1:4	0/4	0/6
1:16	"	"
1:64	1/4	1/6
1:256	4/4	4/6
Normal serum	"	6/6
Virus alone	"	"

* Numerator = No. of tubes showing cytopathic effect or No. of deaths in mice. Denominator = Total No. of tissue cultures or mice.

illustrate type and intensity of cellular changes.

Repeated passage of virus in mouse muscle tissue cultures using a 100-fold dilution of the fluid from preceding culture resulted in reproduction of the cytopathic effect. Increase in rapidity of cellular destruction was noted, complete degeneration occurring in about 48 hours.

Mixing of virus and rabbit immune serum resulted in complete elimination of the cytopathic effect and in failure of agent to kill mice following intraperitoneal inoculation. The results are presented in Table I.

Fig. 3 illustrates growth curve of virus in muscle tissue cultures. After an initial drop in titer, the quantity of virus reached a maximum at 48 hours. Control tubes containing virus plus medium but no cells were free of detectable infectivity for mice after 48 hours incubation at 37°C.

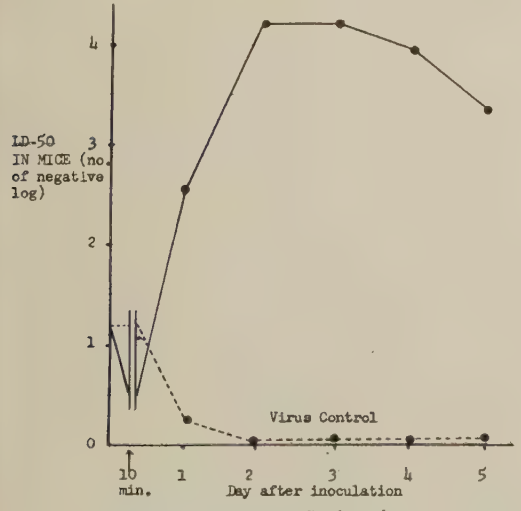

FIG. 3. Growth curve of EMC virus in mouse muscular tissue.

Discussion. Our observations indicate that encephalomyocarditis virus grows well in cultures of abdominal muscle of mice. Although this agent has been propagated in other types of cells, the method described here has the advantage of employing tissues of an animal, almost always available in microbiological laboratories and does not require constant propagation of a cell line such as with HeLa cultures, or use of material obtained from humans or monkeys.

The question may be raised whether the cytopathogenic effect observed, was due directly to encephalomyocarditis virus or to activation of a latent agent present in mouse muscle and carried along in serial cultures. Complete inhibition of cell destruction by addition of specific antiserum suggests strongly that the tissue changes were due to invasion by the virus being studied and not by some unknown organism.

Conclusions. 1. Encephalomyocarditis virus grows readily in cultures of abdominal muscle of mice. 2. A characteristic cytopathic effect is produced; complete destruction of cultures occurs in about 72 hours. 3. The cellular changes are not due to activation of a virus latent in mouse muscle but to the agent of encephalomyocarditis.

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Received June 22, 1959. P.S.E.B.M., 1959, v102.

Andro-stanazole, A New Orally Active Anabolic Steroid. (25184)

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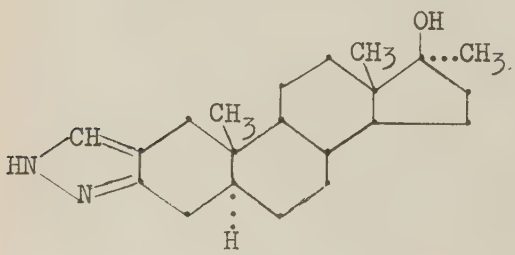
Testosterone and its esters have been used clinically as anabolic agents(1,2) but usage has been severely limited by early appearance of undesirable masculinization. Numerous steroids have been synthesized in search of an anabolic agent, preferably active by oral administration, which would have a more favorable ratio of anabolic to androgenic activity. Estimation of anabolic activity in rats has usually been based upon quantitative increase in weight of the levator ani muscle(3,4) or a decrease in urinary nitrogen(5). We have utilized the sensitivity of castrated male rats to anabolic agents for quantitative evaluation of nitrogen retaining properties(6) as well as for measurement of androgenic activity. It has been found that 17β-hydroxy-17α-methyl-androstano-[3,2-c] pyrazole (I, hereafter referred to as andro-stanazole), described by Clinton *et al.*(7), has pronounced anabolic properties(8) with a relative reduction of androgenic activity when administered orally or parenterally. In the following paragraphs, the anabolic and androgenic activities of andro-stanazole are compared with established reference androgens.

Procedure. (1) As suggested by Kochakian (6), 200 g castrated male rats were brought essentially to weight and nitrogen equilibrium. The initial feed allotment of 14 to 15 g per day was decreased gradually until body weights of the rats reached a plateau. The rats were used after 3 to 4 months at which time the feed allotments were 10 to 11 g per day. In general, the prepared rats were used only once in 6 weeks, and under these conditions they have been utilized up to 2 years. The diet used was composed of: cerelose 33.67, dextrin 33.67, yeast (Fleischmann's 2019) 9.2, hydrogenated vegetable oil (Primex) 7.4, lactalbumin 6.0, methyl cellulose 5.0, salt mixture(9) 3.7, choline dihydrogen citrate 0.9, cod liver oil (2000 A, 250 D) 0.25, wheat germ oil 0.16, and liver extract (Wilson's fraction 0) 0.05. Bulk was supplied by adding 5 parts alphacel per 100 parts diet. This diet supplied 1.4% nitrogen, equivalent to 9.35% protein. At feed intake of 10 to 11 g per day the rats received 150 to 175 mg nitrogen per rat. Urinary nitrogens of the experimental animals generally averaged 100 mg per rat per day with individual values varying 10-15 mg of nitrogen per rat per day on either side of the group average. Fecal nitrogens were not determined because they are constant under conditions of daily weighed feed allotments(1,2). The rats were housed in metabolism racks, and control urinary nitrogen values were determined over a 3-day collection period (Sunday to Wednesday). The urine and daily cage washings were filtered through glass wool into collecting bottles, and samples were diluted to 500 ml preparatory to macro-Kjeldahl nitrogen determinations(10) using 25 ml aliquots. Premedication urinary nitrogen excretion values and body weights provided the basis for distribution of rats with urinary nitrogen excretion of 85 mg of nitrogen or more into uniform groups. The test compounds were administered over a 5-day period (Sunday to Thursday) following the control period. Urine collections were made over the last 3 days of the medication period (Tuesday to Friday) to permit 2 days for response. (2) Androgenicity of the test agent was evaluated by its effect on weight of the ventral prostate gland of 22 day-old castrated rats(11). Starting 7 days after castration, the test materials were administered subcutaneously or



(I)

TABLE I. Relative Effectiveness of Andro-stanazole, Testosterone Propionate and Methyltestosterone on Nitrogen Retention by Castrated Male Rats.

Compound	Dose, mg/rat/day	No. of animals	Body wt, g		Urinary N, mg/rat/day			Relative activity
			Initial	Final	Pre-test	Test	Retained	
Methyltestosterone (oral)	6.0	4	274	278	109.6	103.9	5.7	
	24.0	7	282	285	112.0	97.8	14.2	1
Andro-stanazole (oral)	.1	4	275	277	106.9	104.0	2.9	
	.4	6	285	289	110.7	99.9	10.8	
	1.6	7	277	282	112.0	91.2	20.8	
	6.4	6	269	273	113.6	94.1	19.5	30
Andro-stanazole (s.c.)	.1	4	254	257	111.6	108.4	3.2	
	.4	7	275	279	111.8	97.9	13.9	
	1.6	5	266	271	113.2	95.8	17.4	
	6.4	7	281	290	114.6	89.3	25.3	30
Methyltestosterone (oral)	2.0	8	323	326	96.2	84.5	11.7	
	6.0	7	316	321	98.9	84.4	14.5	
	18.0	6	301	303	99.5	80.4	19.1	1
Andro-stanazole (oral)	.2	6	326	328	100.2	84.7	15.5	30
Testosterone propionate (s.c.)	.025	6	292	298	96.4	81.6	14.8	
	.079	6	298	302	103.1	83.2	19.9	
	.25	6	292	298	101.8	73.2	28.6	1
Andro-stanazole (s.c.)	.45	7	294	295	100.0	86.7	13.3	
	1.42	7	291	293	100.3	79.8	20.5	.05
Testosterone propionate (s.c.)	.02	6	305	309	98.4	93.6	4.8	
	.08	4	314	320	97.4	78.3	19.1	
	.32	4	296	302	104.9	75.3	29.6	1
Andro-stanazole (s.c.)	1.0	5	302	306	98.7	83.3	15.4	.06
Testosterone propionate (s.c.)	.02	5	290	291	98.8	92.0	6.8	
	.08	6	294	296	98.2	82.3	15.9	
	.32	7	299	302	99.4	68.1	31.3	1
Andro-stanazole (s.c.)	1.0	7	299	299	99.1	87.9	11.2	
	4.0	6	300	302	101.2	80.8	20.4	.05

orally for 5 days. The vehicle was cottonseed oil, containing ethanol, 10% W/V. The daily dose was contained in 0.2 ml of vehicle when injected subcutaneously or in 0.4 ml when given by stomach tube. The rats were sacrificed on the sixth day, 24 hours after the last medication. The ventral prostates were excised, blotted and weighed on a micro-torsion balance, and compared with the organ weight of appropriate control animals.

Results. The nitrogen-retaining activity of andro-stanazole was compared with that of methyltestosterone by oral administration and testosterone propionate by subcutaneous administration (Table I). On the basis of the log dose : response formula(12), andro-stanazole orally was 30 times more active than methyl-testosterone in effecting nitrogen retention; subcutaneously, andro-stanazole was one-twentieth as active as testosterone propionate. In general, andro-stanazole was about as effective orally as parenterally. This suggests that andro-stanazole becomes available to the organism as rapidly following oral administration as following parenteral administration.

As indicated by Kochakian(6) castrated male rats are quite sensitive to the effects of parenterally administered testosterone propionate. They respond poorly to orally administered methyltestosterone, which cannot be evaluated at higher levels in this test procedure because the rats refuse to eat when the dose is increased above 20 to 25 mg per day. The adverse effect of methyltestosterone at elevated dose levels has been noted by others (13).

The results of comparative tests of the androgenicity of andro-stanazole, methyltestosterone and testosterone propionate are sum-

TABLE II. Relative Androgenic Activities of Andro-stanazole, Methyltestosterone and Testosterone Propionate. 6 rats/group.

Compound	Dose, mg/kg/day	Body wt, g		V. prost. wt, mg	Relative activity
		Initial	Final		
None		77	106	7.3	
Andro-stanazole (oral)	42.0	76	97	20.2	
	84.0	77	97	31.3	
	168.0	"	89	40.5	.25
Methyltestosterone (oral)	10.5	78	107	17.4	
	21.0	77	104	31.4	
	42.0	"	102	42.7	1
None		77	106	5.7	
Andro-stanazole (s.c.)	7.0	76	107	13.6	
	14.0	77	108	24.9	
	28.0	76	102	27.5	.025
Testosterone propionate (s.c.)	.175	"	107	15.9	
	.35	"	109	20.5	
	.70	"	107	28.0	1

marized in Table II. Over comparable ranges of ventral prostate responses, andro-stanazole was about ¼ as androgenic as methyltestosterone when each was given orally. Parenterally andro-stanazole was about 1/40 as androgenic as testosterone propionate. Andro-stanazole was about 3 times as androgenic given parenterally as orally.

Discussion. For guidance in seeking evidence of separation of anabolic activity from androgenic properties of steroids, the quantitative retention of urinary nitrogen by medicated equilibrated rats was deemed to be a more specific index of anabolic action of an androgen than is the increased weight of the levator ani muscle. On the basis of comparative data obtained in rats, it was inferred that andro-stanazole may be clinically useful as an orally active weak androgen with marked anabolic activity. Howard, Norcia, Peter and Furman(14) have confirmed that this compound, administered orally at varied dose levels to androgen sensitive patients, is capable of inducing significant retention of urinary nitrogen without appearance of masculinizing side effects under the conditions of their experiment. The known properties of this compound suggest that it will be possible to obtain significant conservation of protein nitrogen with oral doses sufficiently small that androgenic side effects will be absent in short-term regimens, and delayed as well as minimal under conditions of more chronic administration.

Summary. Andro-stanazole (17β-hydroxy-17α-methylandrostano[3,2-c] pyrazole) has been evaluated for its nitrogen-retaining and androgenic activities in castrated male rats. Orally, it appears to be 30 times more anabolic and ¼ as androgenic as methyltestosterone. Parenterally, it appears to be 1/20 as anabolic and 1/40 as androgenic as testosterone propionate. Andro-stanazole is clearly an agent which merits further investigation for oral administration to patients under conditions where maximum anabolic action with minimum androgenic side effects is desired.

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Received June 24, 1959. P.S.E.B.M., 1959, v102.

Demonstration of Gamma Globulin in Vascular Lesions of Experimental Necrotizing Arteritis in the Rat.* (25185)

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In renal and vascular lesions of human periarteritis nodosa, presumably of antigen-antibody character, gamma globulin has been demonstrated by fluorescence microscopy(1). In a few instances of other human and experimental arteritis of similar origin, gamma globulin has also been demonstrated(2,3). It appeared therefore interesting to look for gamma globulin in the walls of vessels with necrotizing arteritis, which is not considered to be due to immunological causes. Necrotizing arteritis was produced in rats by unilateral nephrectomy followed by administration of DOCA(4). These lesions were examined for presence of gamma globulin(5,6).

Material and methods. Thirty-nine female Sprague-Dawley rats weighing approximately 150 g were divided into 3 groups: *Group 1,* consisting of 16 rats, were subjected to unilateral nephrectomy under nembutal and ether anesthesia. Subsequently they were given 1.5 mg of DOCA† intramuscularly 3 times a week for 5 weeks. The dosage of DOCA was then increased to 2.5 mg daily. One % NaCl was provided for drinking water. The diet consisted of standard pellets. This regimen was continued for varying periods to 7½ months after which the animals were sacrificed. From some animals, heart blood was drawn for determination of serum gamma globulin(7) immediately before death. The animals were weighed at beginning, occasionally during, and at end of experiment. Weight loss and a general deterioration, reflected in ruffled fur, lethargy and loss of appetite, were used as criteria for selecting time of sacrificing. Some rats succumbed spontaneously. *Group 2* consisted of 13 animals treated as Group 1 but sacrificed within the first 5 weeks (before increase in dosage of DOCA). Eight died spontaneously within 7 to 10 days after operation. *Group III* consisted of 10 rats which only had unilateral nephrectomy and received an ordinary diet and tap water. Immediately after death, tissues were removed from all organs except central nervous system and skeleton, and prepared in the following ways: Blocks of tissue were fixed in 10% neutral formalin for preparation of paraffin sections. Blocks from various organs were snap-frozen at –70°C in a dry ice-isopentane mixture, stored at –30°C and sectioned in a cryostat at approximately 4 μ. Both paraffin and cryostat sections were stained by the conventional staining methods. Staining with fluorescein-labelled serum was carried out, generally following the method of Coons(5,6): Rabbits were immunized with rat gamma globulin (RGG)(8) and bled from the heart. Gamma globulin was precipitated out with 18% Na_2SO_4(9), purified and conjugated with fluorescein-isothiocyanate(10,11). Cryostat sections of rat tissues were then stained with this labelled rabbit anti-RGG serum globulin solution. Controls included: (1) testing the system with non-fluoresceinated rabbit immune serum for blocking of the reaction, and (2) testing the system with normal rabbit serum

* This work supported by research grant from Block Fn.

† Some of the DOCA was provided through kindness of Ciba Pharmaceutical Products.

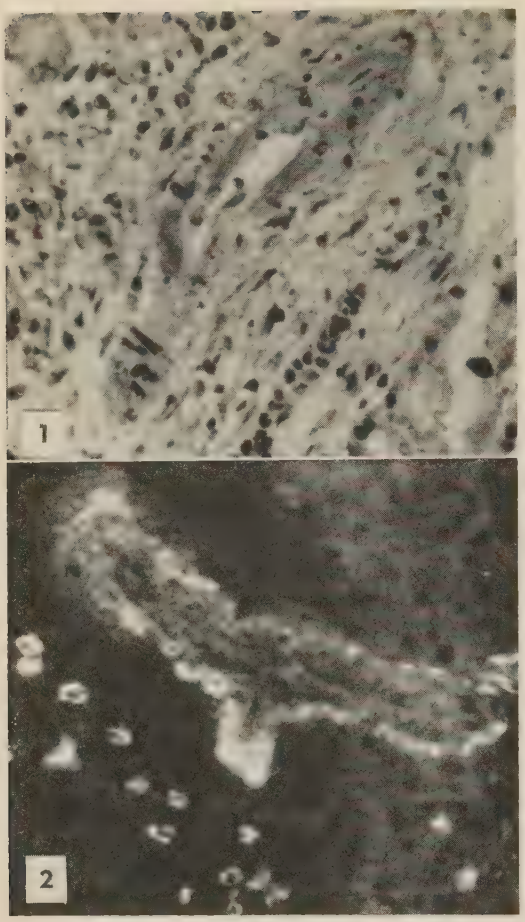

FIG. 1. Small artery in pancreas showing acute necrotizing arteritis with fibrinoid deposits in the media. Cryostat section stained with hematoxylin and eosin.

FIG. 2. Small artery similar to the one shown in Fig. 1. Stained with fluorescein-labelled anti-rat gamma globulin. The fluorescence appears localized chiefly in smooth muscle of media. Plasma cells in environment of involved artery also show fluorescence.

for failure to block the reaction. Fluorescein-stained sections were examined in Zeiss fluorescence microscope using BG 12 filter between light source and specimen, and CG5 filter between specimen and ocular.

Results. Animals in Groups II and III had no demonstrable lesions. In Group I, gross lesions were present in about one-fourth of the animals. Microscopically in large vessels, predominantly splanchnic lesions varied from slight, early swelling of the intima to granuloma formation after 3 months. In the fully developed arteritis, three zones could be recognized: A necrotic inner zone, a granulomatous intermediate zone with radially arranged epithelioid cells and an outer zone of circular collagenous fibres and fibroblasts. Undamaged vessels, when stained with fluorescein-labelled anti-gamma globulin, had no fluorescence. Those blood vessels which had undergone complete necrosis had a diffuse brilliant green fluorescence of the necrotic vessel walls, which was not blocked by the unconjugated anti-rat gamma globulin. The greatest intensity of this fluorescence was in the outermost zone. In those vessels where the structure was still partially preserved (Fig. 1), fluorescence was more focal and could be blocked partially by unconjugated rabbit anti-RGG. The fluorescence was distinctly localized in the damaged smooth muscle (Fig. 2). This was particularly striking where the architecture of the muscle was still recognizable, that is, probably in the milder or earlier phases of the disease process. The smaller the amount of damage in the vessel wall, the more completely could non-specific fluorescence be eliminated. In those arteries presenting a granulomatous reaction, fluorescence was limited to the inner "fibrinoid" zone. This was not entirely specific.

The same degree and extent of fluorescence was noted in small arteries and arterioles, where there was minimal vessel damage, as was noted for the larger arteries.

When sections were stained with anti-rat plasma serum from which the anti-rat globulin had been precipitated out, no fluorescence was obtained in the damaged vessel wall, regardless of degree of necrosis.

In kidneys, besides necrotizing angiitis, lesions were observed in many glomeruli which were extensive but varied in severity and resembled those seen in malignant hypertension in man. The mildest change consisted of increased cellularity of the glomerulus and thickening of the glomerular tuft with hyalinization; the severe change, of partial or complete necrosis of the glomerulus. The proximal convoluted tubules were dilated and filled with large protein casts. In the early glomerular lesions, gamma globulin could be demonstrated in thin, wavy, fragmented fluorescent lines, apparently corresponding to the base-

ment membrane. In more advanced lesions, gamma globulin was also seen in many of the swollen endothelial cells as well as in the precipitated protein within the capillary lumen and in Bowman's space.

In animals which survived 7½ months, and which had not received DOCA for the last 3½ months, no active vascular lesions were found. In only a few of the thickened vessel walls was gamma globulin still present. In the kidney focal glomerulo-fibrosis and cystic dilatation of the tubules without gamma globulin were noted.

Discussion. Localization of specific fluorescence in the vessel wall suggests binding of rat gamma globulin in the damaged smooth muscle. Control experiments with anti-rat plasma rabbit serum from which anti-rat gamma globulin had been removed yielded no specific fluorescence in the necrotic vessels. This would suggest that rat gamma globulin is selectively deposited in the wall of the damaged vessel and that this is not the result of simple diffusion of plasma constituents from the circulating blood into the tissues.

The observation by others of fibrin in vascular lesions of human peri-arteritis nodosa (12) and of serum albumin along with gamma globulin in vascular lesions of experimental serum sickness(3) should caution against hasty conclusions as to the significance of these findings.

In the kidney on the other hand, increased amount of gamma globulin in the more damaged glomeruli may merely reflect altered permeability secondary to severe capillary damage.

Summary. Experimental necrotizing arteritis was produced in female Sprague-Dawley rats by administration of large doses of DOCA and NaCl after unilateral nephrectomy. Presence of RGG in damaged vessel walls was demonstrated by fluorescence antibody technic of Coons. Gamma globulin is deposited in smooth muscle of damaged small arteries, especially in early phases of vascular damage, suggesting that antibody may not be required for this process.

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Received June 25, 1959. P.S.E.B.M., 1959, v102.

Microfluorimetric Evidence of Antigenic Difference Between *Entamoeba histolytica* and *Entamoeba hartmanni*. (25186)

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The complex of amebae known as *Entamoeba histolytica* includes: a large hematophagous form (20-40 μ diameter) associated with acute amebic dysentery; a medium sized form (10-20 μ) generally found in asymptomatic carriers; and a small organism (5-11 μ) generally regarded as never associated with disease(1,2). Burrows(3) recently reviewed the literature dealing with so-called "small race" and, in addition, contributed new morphological data comparing small and medium sized amebae. He concluded that the "small race" was properly a different species, and that its original designation of *Entamoeba*

hartmanni (Prowazek, 1912) was correct. This distinction is of practical, as well as academic, importance since 30 to 50% of human infections assigned to *E. histolytica* are probably due to *E. hartmanni*(1). Asymptomatic infection with the former calls for specific therapy due to its potential pathogenicity, whereas the latter may be ignored. Conventional serologic evidence for the relationship of the 2 forms is meager but, in general, supports the hypothesis of antigenic distinction between the 2 forms(3). Using an immunocytochemical technic, we demonstrated previously that fluorescein-labeled antisera to *E. histolytica* and *Entamoeba coli* stained the homologous species brightly, but stained *E. hartmanni* poorly, providing direct microscopic evidence of antigenic differences among the 3 amebae(4). Staining results were evaluated visually and no objective quantitation of fluorescence was made. The present communication reports use of a microfluorimeter to measure quantitatively the fluorescence of amebae stained with fluorescent antibody, and shows that the results obtained offer quantitative evidence of species distinction between *E. hartmanni* and *E. histolytica*. Complete reports dealing with all phases of this long-term project will be reported.

Materials and methods. Antiserum to strain 22 of *E. histolytica* was prepared in rabbits by inoculating them with amebae growing in culture with an unidentified anaerobic, spore-forming bacterial species. The globulin portion of the serum was labeled with fluorescein (5). Four strains of *E. histolytica* (K-9, M-18, 22 and Huff), and one each of *E. hartmanni* (335), *E. coli* (W28), and a free-living ameba, *Entamoeba moshkovski,* were studied. Ameba smears were prepared by suspending saline-washed organisms from single tubes of 48 hour cultures in 0.15 M acetate buffer, pH 4.7, and drying drops of suspension on to slides. Each smear contained from 1000 to 4000 amebae. Preparations were exposed to 0.1 ml of anti-*E. histolytica* labeled conjugate, and duplicate preparations were exposed to the same volume of normal, labeled rabbit globulin. Prior to this, both solutions had been adjusted by fluorimetric means to the same content of protein-bound fluorescein. After one hour incubation, smears were washed, dried, mounted in glycerol, and examined. Fluorescence intensity measurements were taken of single, entire amebae with the background blocked off by a variable iris diaphragm in the focal plane of the microfluorimeter(6). At the same time, the diameter of each ameba was ascertained, since the diaphragm had previously been calibrated in terms of the microscopic field circumscribed at any setting. A "brightness/unit area" figure was computed for each organism in terms of amperes of phototube current/square μ of ameba. Average brightness of 50 amebae compared staining reactions of the different strains. Since amebae prepared as described above tended to stain to varying degrees even with normal, liver-absorbed conjugate(5), staining results were evaluated in terms of ratio of reaction with anti-*E. histolytica* conjugate to that with normal conjugate. Specificity of staining with 2 of the *E. histolytica* strains was demonstrated in separate experiments by inhibition test of Coons and Kaplan (5) as modified by the author(7).

Results. Pertinent data are summarized in Table I. Average diameters of 25 living ame-

TABLE I. Relative Uptake of Fluorescent Globulin by Seven Strains of Amebae.

Ameba strain	Avg diameter of 25 living amebae	Avg brightness of 50 amebae exposed to labeled E.h. antiglobulin	Avg brightness of 50 amebae exposed to labeled Normal globulin	Ratio of antiglobulin : normal globulin
E. histolytica K-9	18.0 ± 1.6*	4.11 ± .36	1.23 ± .28	3.3
" M-18	15.8 ± 1.6	4.05 ± .40	1.15 ± .12	3.5
" 22	15.0 ± 1.4	3.10 ± .22	.81 ± .14	3.8
" Huff	not done†	3.01 ± .32	1.46 ± .18	2.1
E. hartmanni 335	11.4 ± 1.3	1.31 ± .14	2.79 ± .34	.46
E. coli W28	23.8 ± 1.6	1.97 ± .22	2.95 ± .41	.67
E. moshkovski	15.7 ± .78	.81 ± .07	.95 ± .08	.85

* Confidence intervals given represent twice stand. errors of means.

† Avg diameter of stained amebae of this strain was the same as that of strains M-18 and 22, indicating that living amebae were probably about 15 μ in size.

bae of each strain (except Huff) are presented to show significant size differences between *E. hartmanni* and the 4 strains of *E. histolytica*. Absolute brightness levels of *E. histolytica* strains were considerably higher than those found for other amebae. However, because of the varying uptake of normal conjugate, differences between strains are made more evident by comparing the ratios of brightnesses resulting from reaction with the 2 conjugates. It is clear from these ratios that the 4 strains of *E. histolytica* took up considerably more fluorescent antiglobulin than normal globulin, from 2.10 to 3.8 times as much. On the other hand, the 3 other amebae took up more of the normal conjugate than of the anti-*E. histolytica* conjugate. Of the 4 strains of *E. histolytica*, Huff was significantly different from the other 3 strains, whereas the latter differed from each other insignificantly.

Discussion. The magnitude and consistency of the differences shown between 4 known *E. histolytica* strains and 2 large, non-*histolytica* species, *E. coli* and *E. moshkovski*, are a strong indication that true antigenic differences are being measured by this technic. The fact that the small ameba, *E. hartmanni*, falls definitely within the non-*histolytica* type of reaction is evidence that it, too, is antigenically distinct from *E. histolytica*.

The difference between *Eh*-Huff and the 3 other strains of *E. histolytica* correlates well with invasiveness of the strains in hosts from which they were originally obtained. *Eh* K-9, M-18 and 22 were all isolated from patients with active amebic dysentery or disseminated amebiasis. *Eh*-Huff was the only strain derived from a non-symptomatic individual who carried the infection without apparent ill effects for several years(8). Thus, there is indication here of a relationship between invasiveness and antigenic character. The bacterial associates of these strains and *E. hartmanni* were non-defined but probably similar, since about 2 weeks before the experiment was performed, an emulsion from the same stool specimen was added to them to stimulate growth of the amebae.

It is obviously necessary to extend these observations before making a final commitment, but it appears that use of *"Entamoeba hartmanni"* to designate the so-called "small race-*histolytica*" may be considered to have a serologic basis in addition to the other bases described in the literature. Furthermore, the microfluorimetric approach used in this work may make it possible to recognize different serotypes within the same species, thus contributing to a better understanding of amebiasis.

Summary. Fluorescence of 7 ameba strains was measured with a microfluorimeter following their exposure to fluorescent anti-E *histolytica* and fluorescent normal globulin. Four strains of *E. histolytica* were similar to each other, but were markedly different from *E. coli*, *E. moshkovski* and so-called "small race *E. histolytica*" = *E. hartmanni*. In the *E. histolytica* group, 3 originally invasive strains differed significantly from one non-invasive strain.

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Received June 29, 1959. P.S.E.B.M., 1959, 102.

Effect of Chlorpromazine on Cyanide Intoxication.* (25187)

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Guth and Spirtes found that chlorpromazine and other phenothiazines antagonized cyanide intoxication in pigeons, but they were unable to account for this effect by methemoglobin formation, bypass of enzymes, competition for enzyme sites, complex formation or inhibition of mitochondrial permeability(1). However, a number of drugs protect against anoxia by lowering body temperature(2). The present experiments indicate that chlorpromazine antagonism of cyanide (histotoxic anoxia) can be explained also by occurrence of hypothermia, at least in the species studied.

Methods. Hydrogen cyanide was used because the respiratory route permits control of rate of absorption and because surviving rats show brain lesions in high incidence(3,4). It was generated by passing air through 100 cc of 5% potassium cyanide solution(4). Rats (female albino, 150-250 g) or mice (female C57-leaden, 20-30 g) in weight matched pairs were exposed in gallon or pint jars (rats and mice differ in rate of heat loss under hypothermic conditions because of different surface-volume ratios). One member of each pair was pretreated with intraperitoneal chlorpromazine (50 mg/kg); the control was sham-injected or given an equal volume of saline. In some experiments (mice and rats) hydrogen cyanide was administered at rates that were varied in accordance with the condition of the animals so as to produce fatalities over a wide range of time (from a few minutes to 1½ hours); as soon as one member of each pair died the other was removed and sacrificed a few days later for histologic study. In other experiments (mice) the flow was kept at a constant rate of 0.1 liter/minute/jar and hydrogen cyanide vapor was delivered until both members of all pairs were dead (judged by cessation of respiration); the time till death was recorded.

Results. Eighteen pairs of mice were exposed to hydrogen cyanide delivered at various rates that produced fatalities over a wide range of time (5-83 minutes). In all but 2 pairs, the control died first, showing the protective effect of chlorpromazine pretreatment.

Twenty pairs of mice (in 4 groups of 5 pairs each) were exposed to hydrogen cyanide delivered at a constant rate. Two groups were kept in air, and the chlorpromazine-pretreated mice showed 2.8°C average drop in rectal temperature in the 15 minutes before exposure. In the other 2 groups, chlorpromazine-induced hypothermia was prevented by keeping the jars in 36°C water bath for 15 minutes between injection and exposure, and during entire exposure. Table I shows that chlorpromazine nearly doubled survival time in groups with lowered body temperature, while the protective effect was largely eliminated when body temperature was maintained at normal levels.

Twenty-three pairs of rats received hydrogen cyanide at rates deliberately varied so as to produce fatalities over a wide range of time (14-75 minutes). In contrast to results with mice, the outcome depended on rate of cyanide administration. The control rat died first in 13 pairs; average time was 26.2 minutes. The chlorpromazine-pretreated rat died first in 10 pairs; average time was 54.5 min-

TABLE I. Effect of Chlorpromazine on Cyanide Intoxication, with and without Preliminary Temperature Drop.

	Chlorpromazine		Control	
	△ Temp. (°C)	Survival (min.)	△ Temp. (°C)	Survival (min.)
1*	−2.1	21.0	.0	10.8
2*	−3.5	21.2	− .1	12.0
3†	+ .1	13.6	.0	11.2
4†	+1.3	14.4	+1.0	11.0

* Groups 1 and 2 in air to permit temperature drop after chlorpromazine.

† Groups 3 and 4 in 36°C water-bath to prevent temperature drop.

* This investigation was supported wholly by Grant from Nat. Multiple Sclerosis Soc. Chlorpromazine was kindly supplied by Smith, Kline & French Lab., Philadelphia, under trade name Thorazine.

TABLE II. Effect of Chlorpromazine Pretreatment on Rats Exposed to Hydrogen Cyanide.

First fatality*	Time†	First fatality*	Time†
Control	14	Control	36
CPZ	15	CPZ	39
Control	18	Control	41
Control	20	CPZ	45
Control	21	CPZ	50
Control	22	CPZ	54
Control	22	CPZ	63
Control	25	CPZ	64
Control	27	CPZ	67
Control	30	CPZ	73
Control	32	CPZ	75
Control	33		

CPZ = Chlorpromazine pretreatment.

* The exposure was discontinued after death of one rat from each pair, and the survivor was saved for histologic study.

† The hydrogen cyanide was delivered intermittently and at various rates in order to have death times over the range of 14 to 75 min.

utes. Apparently chlorpromazine failed to protect rats against slow hydrogen cyanide administration (Table II). Examination of surviving chlorpromazine-pretreated rats showed cyanide brain lesions in corpus callosum, corpus striatum, cerebral cortex, hippocampus, thalamus and substantia nigra. These lesions were similar in type and distribution to those in control rats and to those reported previously(3).

Chlorpromazine-pretreated mice and rats showed depressed respiration which disappeared with beginning of cyanide administration; they underwent the same stages of hyperpnea, automatic respiration and terminal respiratory depression as the controls.

Discussion. Antagonism of cyanide intoxication by chlorpromazine, reported by Guth and Spirtes for pigeons(1), has been confirmed for mice. The protective effect was associated with decreased body temperature, and was largely eliminated when normothermia was maintained. Chlorpromazine pretreatment of rats failed to protect against slow, protracted hydrogen cyanide exposures. The explanation may depend on 2 factors: (a) chlorpromazine produced less pre-exposure drop in body temperature in rats than in mice, probably due to larger bulk of rats, and relatively warm room temperature; (b) profound cyanide intoxication caused a drop† in body temperature(5), proportional to depth and duration of poisoning, and this tended to level off temperature differences between chlorpromazine-pre-treated and control rats as the exposure progressed.

Chlorpromazine, like cyanide, has been reported to inhibit cytochrome oxidase(7), therefore a synergistic rather than antagonistic effect might be expected. The observed antagonism shows that cytochrome oxidase inhibition does not dominate the pharmacologic action of chlorpromazine, but it may become significant when the more important hypothermic effects of the drug are reduced or eliminated.

The antagonism of cyanide intoxication by chlorpromazine, according to our interpretation, is analogous to protection against anoxic anoxia (low atmospheric pressure) by Megaphen (a phenothiazine derivative), cardiazol, pyramidon, ethyl alcohol and other drugs reported by Flacke *et al.*(2). In their experiments, as in ours, protection was lost when temperature-lowering was prevented. It is well known that hypothermia protects the brain against anoxia, and we found, for example, that cooling anesthetized rats till their temperature was 25°C enabled them to withstand up to 2 hours a flow of hydrogen cyanide that killed normal rats in 10-20 minutes.

Summary. Hydrogen cyanide intoxication is antagonized by chlorpromazine in mice and, to a lesser extent, in rats. The protective effect of chlorpromazine is due, in large part, to hypothermia induced by this drug.

† LeBlanc studied the interaction of cyanide and chlorpromazine and reported that cyanide did not affect body temperature of rats(6). The dose of cyanide was much less than the amount required for deep intoxication, therefore this finding is not in conflict with our observations and those of Dervillée *et al.*(5).

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Received July 6, 1959. P.S.E.B.M., 1959, v102.

Effect of Neomycin, Para-Aminosalicylic-Acid and Other Antibacterial Drugs on Serum Cholesterol Level of Man. (25188)

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It has been reported(1-2) that oral administration of neomycin lowered serum cholesterol concentration significantly in 10 patients. In extending this study, the effect of neomycin and other antibacterial drugs upon serum lipid levels of an additional group of patients was observed.

Methods. Twenty-five patients, 14 males and 11 females, were studied in 44 experimental periods. The average age was 54.2 years (35 to 74 years). Sixteen patients were hospitalized and 9 were followed as outpatients. Clinical diagnoses were coronary artery disease (6 patients), cerebral vascular disease (8 patients), familial hypercholesterolemia (2 patients), familial hyperlipemia with coronary artery disease (1 patient), diabetes mellitus (2 patients, including one of the CVA patients). In 7 patients there was no evidence of vascular disease or disturbance of lipid metabolism. Serum cholesterol levels were determined once a week, in the fasting state, by the method of Zak *et al.*(3). Serum phospholipid determinations were carried out by the method of Simonsen *et al.*(4) and the alpha and beta cholesterol levels by the method of Langan *et al.*(5). The control serum cholesterol levels, prior to administration of the drugs, were followed during a period of 6 weeks or longer. Hospitalized patients were maintained on regular hospital diet with the exception of the 2 diabetics, and the diet of the outpatients remained relatively constant during the study. A preparation containing 70% of neomycin sulfate (Mycifradin Sulfate,®)† was given orally to 18 patients in doses of 1.5 to 2 g daily for 4 to 20 weeks. Doses of medication in this study represent the weight of Mycifradin Sulfate. Five patients were given 60 mg of neomycin intramuscularly daily for a period of 3 weeks. This amount is equivalent to or higher than the proportion of oral dose (3%) which is absorbed from the gastrointestinal tract(6). Para-aminosalicylic-acid‡ (PAS) was given orally to 4 patients in 6 experimental periods. Four were given 6 g of PAS daily for 4 to 5 weeks. Two patients received 12 g of PAS for 9 and 4 weeks respectively. Isoniazid‡ (300 mg daily) was administered orally to 3 patients for a period of 4 weeks. Phthalylsulfathiazole§ (12 g daily) oxytetracycline‖ (1 g daily), polymyxin B sulfate‖ (150 mg daily) and novobiocin§ (1 g daily) were each given orally to 2 patients for a period of 3 weeks. Dihydrostreptomycin‖ (2 g daily) and bacitracin‖ (20,000 units daily) were each administered orally to 2 patients for a period of 2 weeks.

Results. The results of oral administration of neomycin are summarized in Table I. Mean serum cholesterol levels were lowered significantly in each patient by 17 to 29% during administration of neomycin, the average for the group being 21%. Two to 3 weeks were necessary before the concentration of serum cholesterol decreased to its low point. Cholesterol levels remained low as long as

* This work was done during tenure of Research Fellowship of Am. Heart Assn.

† Supplied by Upjohn Co.

‡ Supplied by Lilly Labs.

§ Supplied by Merck Sharp and Dohme Labs.

‖ Supplied by the Pfizer Labs.

TABLE I. Effect of Neomycin and PAS on Average Serum Cholesterol Level (mg/100 cc), in 20 Patients.

Age	Sex	Diagnosis	Medication and dose	Wk on medication	Total serum cholesterol		
					Control	Medication	% fall
58	♀	CVA	Neo.* 2 g orally	20	355	273	23
55	♂	"	" 2 "	15	314	245	20
42	♂	Coronary artery disease	" 1.5 "	13	342	284	17
52	♀	Diabetes mellitus	" 2 "	10	339	269	20
48	♀	Familial hypercholesterolemia	" 1.5 "	9	513	378	26
68	♂	Coronary artery disease	" 1.5 "	8	293	232	21
42	♂	CVA	" 2 "	8	268	223	17
44	♂	Paraplegia of undetermined cause	" 2 "	8	264	210	20
68	♂	Coronary artery disease	" 1.5 "	7	274	227	17
44	♂	*Idem*	" 1.5 "	7	256	211	17
59	♀	Familial hypercholesterolemia	" 1.5 "	6	438	321	27
46	♀	CVA. Diabetes mellitus	" 2 "	6	304	251	17
41	♀	Multiple sclerosis	" 2 "	5	190	142	25
60	♀	CVA	" 2 "	5	394	281	29
61	♂	Coronary art. dis. Familial hyperlipemia	" 2 "	5	305	249	18
58	♀	No clinical disease	" 2 "	4	313	254	19
63	♂	Coronary artery disease	" 1.5 "	4	273	203	26
35	♂	Multiple sclerosis	" 2 "	4	158	120	24
52	♀	Diabetes mellitus	" 60 mg intramusc.	3	339	322	
42	♂	CVA	" 60 "	3	268	260	
44	♂	Paraplegia of undetermined cause	" 60 "	3	264	269	
46	♀	CVA. Diabetes mellitus	" 60 "	3	304	306	
35	♂	Multiple sclerosis	" 60 "	3	158	168	
61	♂	Coronary art. dis. Familial hyperlipemia	PAS 12 g orally	4	305	226	26
43	♀	Rheumatic heart disease	" 12 "	9	244	168	24
44	♂	Paraplegia of undetermined cause	" 6 "	5	264	245	
61	♂	Coronary art. dis. Familial hyperlipemia	" 6 "	4	305	299	
43	♀	Rheumatic heart disease	" 6 "	4	244	235	
51	♂	CVA	" 6 "	5	242	230	

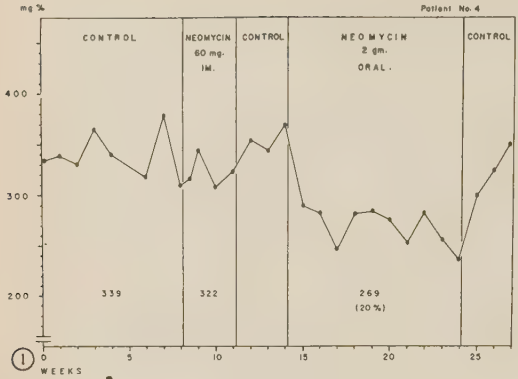
* Neomycin.

the drug was given orally, and returned to control levels 2 to 7 weeks after administration of neomycin was discontinued (Fig. 1 and 2). In 8 patients studied the over-all average of the esterified fraction of cholesterol was 77% during the control period and 78% during administration of neomycin. The mean phospholipid concentrations remained unchanged in 1 case and decreased by an average of 24% in 6 cases during the experimental period in the 7 patients studied. In 6 patients, mean concentration of beta cholesterol decreased in 4 by an average of 9% and remained unchanged in 2.

The results of intramuscular administration of neomycin are included in Table I. In the 5 patients studied, serum cholesterol levels failed to show appreciable changes following intramuscular administration of the drug. (Fig. 1).

No significant side effects occurred in the group given neomycin. A mild, transitory diarrhea was present in 6 of the 18 patients at the initial phase of oral administration of the drug. Weight of the patients remained approximately unchanged.

Table I shows the results obtained with the use of oral PAS. At the daily dose of 6 g of PAS no significant changes in serum cholesterol concentration occurred. Increasing the daily dose of the drug to 12 g in 2 patients resulted in a reduction of average serum cholesterol concentration within 2 weeks by 24% in one and 26% in the other (Fig. 2), with a return to control levels 2 weeks after administration of PAS was discontinued. No significant side effects were noted. A mild nausea



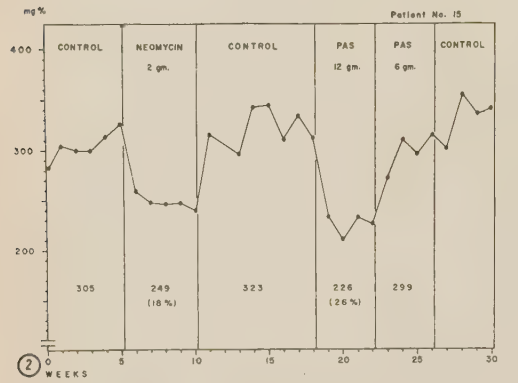


FIG. 1. Effect of intramuscular and oral neomycin on serum cholesterol concentrations.

FIG. 2. Effect of oral neomycin and PAS on serum cholesterol concentrations.

was periodically observed without appreciable changes of the patients' weight.

Oral administration of isoniazid, phthalylsulfathiazole, oxytetracycline, polymyxin B sulfate, novobiocin, dihydrostreptomycin and bacitracin failed to alter serum cholesterol levels appreciably.

Comment. It is known that approximately 97% of orally administered neomycin is eliminated unchanged in the stool and only about 3% is absorbed(6). Since intramuscular administration of 3% or more of the orally effective dose failed to alter serum cholesterol levels, it is apparent that the cholesterol lowering effect of neomycin is dependent upon its action in the gastrointestinal tract. At present the mechanism of this action is not understood. However, it is possible that the effect of neomycin is due to modification of the intestinal bacterial flora or inhibition of intestinal enzyme systems.

The mechanism of the serum cholesterol lowering action of PAS cannot be explained. Recently, Tygstrup *et al.* have also noted the serum cholesterol lowering action of this drug (7). Although PAS is readily absorbed, one cannot rule out the possibility that its action would be mediated *via* changes in the gastrointestinal tract. Studies are in progress to elucidate these problems.

Summary. 1) Neomycin was given orally to 18 patients at daily dose of 1.5 to 2 g for 4 to 20 weeks. Mean serum cholesterol levels were decreased significantly in each patient by 17 to 29%, and were maintained low for duration of administration of neomycin. Average fall for the group was 21%. Intramuscular administration of neomycin to 5 patients for 3 weeks failed to alter serum cholesterol levels. 2) Oral administration of 12 g of para-aminosalicylic-acid to 2 patients lowered serum cholesterol concentrations significantly in each, while the daily dose of 6 g had no effect on serum cholesterol levels. 3) When phthalylsulfathiazole, isoniazid, dihydrostreptomycin, oxytetracycline, polymyxin B sulfate, bacitracin and novobiocin were given orally, no appreciable changes in serum cholesterol concentration resulted.

The cooperation of Drs. Michael M. Decso, Menard M. Gertler and staff of Department of Physical Medicine and Rehabilitation, N. Y. University, Goldwater Memorial Hospital, for making available patients for study, is gratefully acknowledged.

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Received July 7, 1959. P.S.E.B.M., 1959, v102.

A Constant Temperature Tissue Bath. (25189)

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Use of isolated tissue preparations for study of their physiological and pharmacological reactions necessitates some device for maintenance of a stable environmental temperature, usually in the vicinity of 38°C. This is usually accomplished by means of a large reservoir of water containing a thermostat, heater and stirrer; the tissue bath is either immersed in this or water is pumped through a water jacket surrounding the bath. Both systems are expensive, and usually cumbersome because of the large water reservoir required for accurate temperature control.

Method. The tissue bath described here (Fig. 1) is compact, occupies little space and is inexpensive. It consists of a central compartment (a), in which the tissue is to be suspended in an appropriate saline solution, surrounded by a space (b) continuous below with a solvent reservoir (c) and closed above except for an air vent (d). The upper part of this space is surrounded by a condenser jacket (e) with inflow and outflow tubes (f and g). A drainage tube (h) passes from the bottom

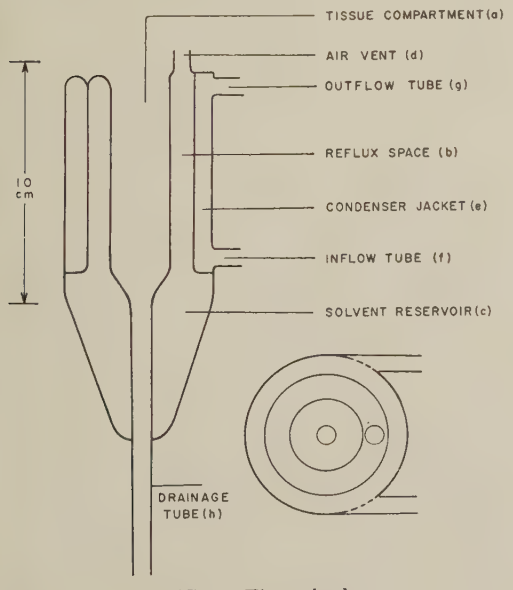

FIG. 1. Tissue bath.

of the central compartment through the solvent reservoir, which is enclosed in a perforated Glass-Col heating mantle designed for hot filtration (110-volt mantle) with top diameter of 2.5 inches. *Rate of reflux* must be controlled to prevent vigorous bumping and rapid dissipation of solvent, both of which will occur with low boiling solvents when the heating mantle is employed at full voltage. The voltage may be conveniently regulated by connecting a light bulb of suitable wattage in series with the mantle.

Temperature of the saline solution may be regulated by a suitable choice of solvent, which should present a negligible hazard from explosion or toxicity. For the majority of experiments it is desirable to maintain the temperature of the saline solution as close as possible to 38°C. With a 100-watt bulb in series with the mantle and employing methylene chloride (b.p. 40.1°C at 760 mm) as the refluxing solvent, a temperature of 38.9°C is obtained for saline solution through which a rapid stream of oxygen is passed. Over a period of 5 hours the variation is $\pm$ .03°C. Commercially available methylene chloride (Matheson catalogue No. 5509) gave consistent results without further purification. Saline solutions must be preheated for bath changes, since approximately a 10-minute period is required to reach the experimental temperature from the initial room temperature of the saline. Preheating may be conveniently accomplished by employing a second tissue bath. For a temperature in the vicinity of 20.0°C an azeotropic mixture of methyl formate and trichloromonofluoromethane (Freon-11, DuPont) may be employed. Temperatures in the range of 24.0°C to 35.0°C may be obtained with an appropriate solution of trichloromonofluoromethane and trichlorotrifluoroethane (Freon-113, DuPont). These 2 compounds do not azeotrope, but when mixed in proper proportions will form a constant boiling solution (Table I).

TABLE I. Temperatures of Saline Solutions with Different Proportions of Freon-11 and Freon-113.

Wt % Freon-11	Wt % Freon-113	B.p. °C
90	10	25.0
80	20	26.4
70	30	28.0
60	40	29.7
50	50	31.7
40	60	34.2
30	70	36.7

Summary. A compact and inexpensive tissue bath enabling the study of isolated biological materials has been developed. Temperature control of the saline solution is achieved by means of a refluxing solvent. When employed at a temperature of 38°C accurate control to ± .03°C is possible. By appropriate choice of solvents it is possible to study isolated tissue preparations over a wide range of temperatures.

Received July 7, 1959. P.S.E.B.M., 1959, v102.

Cytochemical Demonstration of Diphosphopyridine Nucleotide-Diaphorase Activity in Cells of Vaginal Secretions of Mice.* (25190)

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Our previous studies have indicated the presence of at least 2 oxidative enzyme systems in the vaginal epithelium of rodents both of which have been shown to be under the influence of circulating titres of ovarian hormones during normal cyclical events(1,2). The enzyme systems described were the succinic dehydrogenase (SDH) and the diphosphopyridine nucleotide diaphorase (DPN) systems, histochemically demonstrable by using neotetrazolium as an indicator dye. The use of such methods has made localization of oxidative enzyme systems such as these possible at the tissue or histologic level of study. Recently, however, newer more sensitive and suitable tetrazolium salts have been advanced, especially nitro-blue tetrazolium (NBT), which now make possible enzyme localizations at the cellular or cytochemical level of study both with the light microscope(3) and the electron microscope(4). The purpose of this report is to show the cytochemical distribution of one such oxidative enzyme system (DPN) with use of NBT in the cellular population of vaginal secretion during the estrous cycle. It is our intent to show that: (1) cytochemical localization of such oxidative enzymes is possible through use of smear preparations, and (2) that the various cell types resident in vaginal secretions show distinctive enzyme localization patterns each related to degree of cytomorphological change (cornification) in the cell.

Methods and material. Adult, female albino mice (Swiss strain) were used in these studies. The animals were studied through at least 3 consecutive estrous cycles during which time the vaginal lumen was smeared by the use of a cotton-tipped applicator previously moistened in cold Ringer's solution. Smears were prepared immediately on glass slides which were air dried and quickly immersed into the following preheated incubating medium:

.1 M Na phosphate buffer, pH 7.6	14 ml
1.0 M sodium lactate	4 "
Coenzyme I (1 mg/ml aq.)	2 "
Nitro-blue tetrazolium (DAJAC)	20 mg

Following incubation at 38°C for 30 minutes and fixation in 10% neutral formalin, slides were rinsed in water and counterstained in eosin with subsequent mounting in synthetic resin. The insoluble dye, dinitroformazan, precipitated as a result of enzymatic activity has been shown to resist dissolution in the higher alcohols and xylene(3). Under the conditions of these experiments smear preparations incubated in the absence of lactate contained no detectable amounts of dinitroformazan. Parallel studies of each smear were also

* This work supported in part by U.S.P.H.S. Grant.

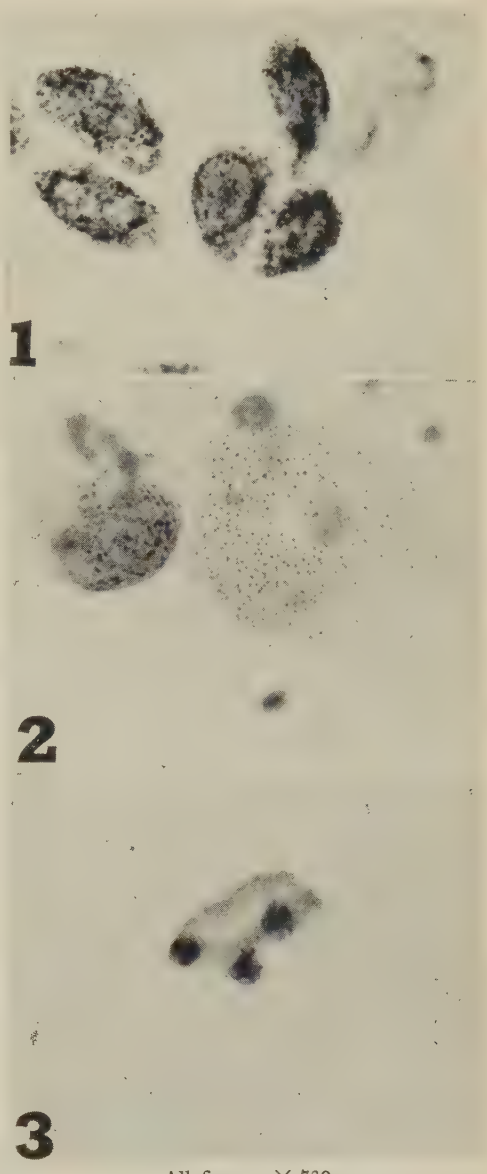
performed using the conventional hematoxylin and eosin method.

Results. Various cells are found in the vaginal secretion and the amount and types of these are dependent upon the stage of the estrous cycle during which the secretion is studied. Briefly, during *estrus* high numbers of well-cornified cells either with degenerate pyknotic nuclei or no nucleus are present. These gradually disappear at *metestrus* when leukocytes invade the vaginal epithelium and reside in the luminal contents. Leucocytes are found in highest numbers in the vaginal lumen during *diestrus* when the titre of circulating estrogen is lowest. Cornified cells are absent and the only other cellular constituent is represented by a nucleated epithelial cell derived from the lining of the vaginal canal at this time. In *proestrus* the leucocytes gradually disappear and the nucleated cells are found in rather high numbers. As the vagina comes under the influence of estrogen and estrus approaches, the nucleated epithelial cells show increasing degrees of cornification. As cornification is completed, nuclear degeneration occurs with subsequent pyknosis and final disappearance of this structure.

Thus, in studying the cellular population of vaginal smears during the different stages of the estrous cycle one can discern 4 main categories of cell types: 1. Cornified, nonnucleated epithelial cells; 2. Nucleated, precornified cells; 3. Nucleated, noncornified cells; 4. Leucocytes, mainly neutrophils. We studied these cells for enzymatic activity; a description follows.

1. *The cornified cell.* The completely cornified cells were devoid of any enzymatic activity (Fig. 1). As cornification progressed the DPN activity which was entirely cytoplasmic continued to decline until no reaction was evident.

2. *Precornified cells.* As indicated above there was no nuclear staining while the cytoplasm of these cells varied in intensity of reaction. Cells which showed the least amount of cornification tended to stain rather uniformly throughout, with granules of dinitroformazan showing a relatively even distribution in the cytoplasm (Fig. 2). As cornification proceeded, lack of staining in the peripheral cytoplasm was evident. Fewer granules were



All figures × 720.

FIG. 1. Noncornified epithelial cells (upper center and left) from an animal in proestrus showing high DPN activity in cytoplasm. Cytoplasmic granulation is so abundant as to obscure the nucleus which normally displays no enzymatic activity. Inactive cornified cell, (upper right).

FIG. 2. Cell at left is in an intermediate stage of cytomorphic change between those found in Fig. 1 and the squamous element next to it. Note dispersion of cytoplasmic granules as cell becomes more cornified.

FIG. 3. Leucocytes containing high DPN activity in association with a portion of a degenerating cornified element.

present, usually of smaller size than those found in earlier stages. In many of these cells DPN distribution was limited entirely to a perinuclear zone which was the last region to show a positive reaction prior to complete cornification and destruction of the enzyme system.

3. *Nucleated, noncornified cells.* These were the most active cellular constituents found during the entire estrous cycle. Rather heavy staining was found throughout the cytoplasm which contained dense accumulations of dinitroformazan (Fig. 1). The granulations in these rounded cells were the largest seen in any of the epithelial cells studied. It should be noted, however, that occasionally some of these cells displayed little or no DPN activity.

4. *Leucocytes.* Most of these cells were of the polymorphonuclear neutrophil type with lymphoctyes and possibly monocytes occasionally present. The majority of neutrophils found in the smear contained little or no enzyme, although in certain preparations, areas of active as well as inactive cells were present. Neutrophils giving a positive reaction were generally associated with degenerating cells and were apparently in the process of active phagocytosis. Here, an intense DPN activity was observed (Fig. 3). Some mononuclear leucocytes also demonstrated heavy cytoplasmic staining.

Discussion. The presence of oxidative enzymes in smear preparations of cells was demonstrated by Hirono who used ascites cells and localized SDH using neotetrazolium as an indicator dye(5). More recently, Marcuse(6,7) studied SDH in endometrial and cervical smears employing the same tetrazole. The use of nitro-blue tetrazolium in similar studies should prove more profitable since the formazan of this dye is resistant to the reagents which allow for permanent mounting of the cells. There is possible, therefore, discrete cytochemical localization of the enzyme and a more sensitive indication of oxidative enzymatic activity. The presence of other enzymes in the cells of the vaginal lavage has also been indicated(8,9). These enzymes, such as the phosphatases and esterases, are not as labile as the oxidative enzymes. The retention of DPN activity in cells divorced anatomically and physiologically from the vaginal epithelium is, therefore, an interesting phenomenon. It is not surprising that cells undergoing an increasing degree of cornification should possess less enzymatic activity inasmuch as this morphologic process is in the nature of a degenerative one. Previous studies employing tissue sections have shown that with increasing cornification DPN is progressively lost in the vaginal epithelium(1,2). In addition, during early proestrus, superficial epithelial cells which contain much mucin are strongly positive for DPN. The present study has shown that the activity is still retained even after exfoliation. The occasional absence of DPN in these cells is probably the result of early death after exfoliation.

Contrary to observations on the basal cells of intact vaginal epithelium, these results indicate that the presence of DPN in the vaginal secretion is lowest during the period of the cycle when estrogen is secreted maximally by the ovary (estrus). This finding has been reported earlier for phosphatases by Ayre in a study of human vaginal smears(6). This worker suggests that degree of enzymatic activity in the cells of the vaginal secretion does not directly depend on estrogen levels in the body but rather on the cytomorphological state of the cells induced by ovarian steroids. The findings in these studies for DPN activity can be interpreted similarly.

The intense activity occasionally seen in certain leucocytes is probably an indication of their increased oxidative metabolism during various phases of activity. Apparently, energy yielding oxidations are required for phagocytic ingestion. We also observed such DPN activity in neutrophils and lymphocytes in smears of human peripheral blood.

Studies similar to those described above have been applied to vaginal smears of humans in normal and pathologic states(10,11). Detailed reports will be published.

Summary. Mouse vaginal smears were studied for diphosphopyridine nucleotide-diaphorase (DPN) activity. Cytochemical findings indicate the absence of enzyme in com-

pletely cornified cells while DPN is increased in cells with less cytodifferentiation.

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Received July 15, 1959. P.S.E.B.M., 1959, v102.

Fibrinolysis III. Nutritional and Environmental Requirements for Optimum Production of Fibrinolysin from Aspergillus (Aspergillin O). (25191)

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Many fungi are known to produce proteolytic substances as part of their metabolic activity and some of these substances are capable of fibrinolysis. Previously reported studies(1) on fibrinolytic activity of extracts from cultures of 260 fungi have singled out a strain of *Aspergillus oryzae* (B-1273) as the most promising producer of material with strong and relatively selective fibrinolytic activity. In continuing these studies, it soon became apparent that further progress would be greatly handicapped by the inability to obtain high yields of material. This paper describes observations on nutritional and environmental requirements of *Aspergillus oryzae* (B-1273) for increased production of fibrinolytic substance. For convenience, this material has been tentatively designated as "Aspergillin O" to distinguish it from the spore pigment, Aspergillin, previously isolated from *Aspergillus niger* (2). Properties and physiochemical characteristics of Aspergillin O are to be described.

Materials and methods. 1. *Culture media.* Czapek's solution agar slants and malt agar slants were prepared according to standard technics from commercial reagents (Difco). The liquid media consisted of following reagents: Sucrose 7.2 g, Dextrose 3.6 g, KH_2PO_4 13.69 g, KNO_3 2.0 g, $MgSO_4$ 1.23 g, in double distilled water to 1 liter. Two hundred ml of the media was transferred to 1 liter Roux culture flasks, then autoclaved 15 minutes at 15 lbs steam pressure (121°C). When needed, salts and vitamins were added to the liquid medium. Stock solutions of trace elements were prepared by dissolving 723 mg $FeSO_4$, 440 mg $ZnSO_4$, and 200 mg $MnSO_4$ in 500 ml of double distilled H_2O. Sufficient volume of concentrated sulfuric acid was added to render solutions colorless and the solutions were then brought to 1 liter with double distilled H_2O. Two ml of each stock solution was added to the medium. Stock solutions of vitamins contained 10 mg of pyridoxine hydrochloride in 100 ml of 20% ethanol solution or 10 mg of thiamine hydrochloride in 100 ml of 20% ethanol solution in water. Other media were prepared in which the source of nitrogen was represented by NH_4NO_3, $NH_4H_2PO_4$, $(NH_4)_2HPO_4$ and KNO_2 rather than KNO_3. These compounds were used in concentrations yielding a nitrogen equivalent to 2 g KNO_3/L. 2. *Fibrin plates.* Solutions of 350 mg % of bovine fibrinogen (Armour) and of bovine thrombin (Parke Davis) containing 30 NIH units/

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ml were prepared in phosphate buffer at pH 7. Nine ml of fibrinogen solution and 1 ml of thrombin solution were quickly mixed in test tube and poured into sterile Petri dish of 10 cm diameter. After clotting, the plates were heated at 85°C for 30 minutes and cooled before use. 3. *Growth of Fungi.* Parent cultures were transplanted to Czapek's solution agar slants, which served as stock cultures and incubated at 24° (± 2) C. Spores from stock tubes were transplanted to malt extract agar slants and cultured at same temperature to provide sufficient quantities of spores for inoculation of the liquid media. This was performed in a single transfer of spores from an agar slant by a sterile platinum loop (1 cm^2 area). Flasks were gently agitated to insure uniform distribution of the inoculum and cultures were incubated 5 days at room temperature in the dark or under different experimental conditions as desired. 4. *Extraction of fibrinolytic material.* The liquid medium was separated from the fungal mat after 5 days of incubation by filtration through filter paper (Whatman 40). The filtrate was passed through a column of IRA-400 resin in the Cl^- cycle. After addition of 1/10 volume of 1/10 N NaOH, the active material was precipitated by addition of 2 volumes of acetone A.G. The precipitate was resuspended in distilled water (2/5 volume of original filtrate) and any insoluble material was separated by filtration through paper. These represent preliminary steps for preparation of Aspergillin O. Full details are given elsewhere. Serial dilutions of the resuspended material from 1:1 to 1:8 were also prepared with distilled water. 5. *Weight of fungal mat.* This was determined on analytical balance after pressing the mat in paper toweling and drying at 80°C for 60 minutes or until constant weight was obtained. 6. *Assay of activity of material.* 0.05 ml of a 1:4 dilution of active material in water was placed on fibrin plates and these were incubated at 37°C for 6 hours. The 1:4 dilution was selected after preliminary work indicated that it would yield uniformly reproducible results, probably because of dilution of contaminating salts and other impurities. One unit of activity was considered equivalent to an area of 10 mm^2 of lysis. *All calculations were made on the basis of original volume of filtrate,* and in the experimental conditions, 0.05 ml of a 1:4 diluted material was equivalent to 1/32 ml of filtrate. The following formula was used for calculations of units of activity:

$$\text{units/ml filtrate} = \left(\frac{\text{lysis area, mm}^2}{10\ \text{mm}^2} \right) \times 32.$$

Results. (Fig. 1). The liquid medium appeared adequate for growth of the fungus, but, in the dilutions used, the material obtained had insignificant fibrinolytic activity. Table I indicates that addition of some trace

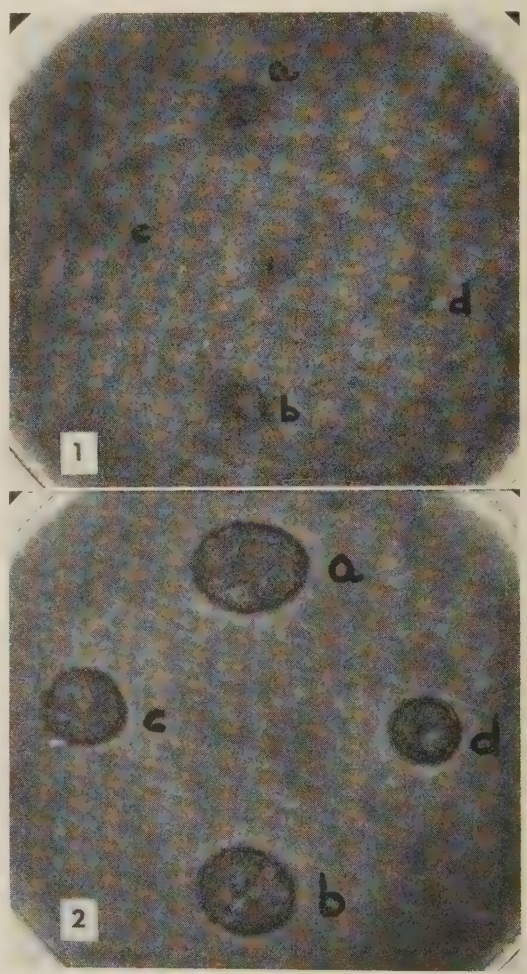

FIG. 1. Lysis of fibrin plates by preparations of Aspergillin O from different cultures. (1) Matèrial from fungi grown in ordinary medium; (2) material from fungi grown in media enriched with trace elements; a, b, c, and d represent yield from .12, .06, .03, .015 ml of original filtrate.

TABLE I. Effects of Trace Minerals and Vitamins on Rate of Growth and Production of Fibrinolytic Material by *Aspergillus oryzae* (B-1273).

Additions	pH		Growth mycelium, dry wt (mg)	Activity, units/ml filtrate
	Initial	5th day		
None	4.3	4.8	56	0
Zn^{++}	"	4.9	286	58
Fe^{++}	4.2	4.7	66	0
Mn^{++}	4.3	"	88	0
Zn^{++}, Fe^{++}	4.2	5.9	787	500
Zn^{++}, Mn^{++}	"	5.4	446	0
Fe^{++}, Mn^{++}	4.3	4.6	74	0
Zn^{++}, Mn^{++}, Fe^{++}	4.2	5.7	753	552
Zn^{++}, Mn^{++}, Fe^{++} Vit. B_1	4.4	5.9	739	552
Zn^{++}, Mn^{++}, Fe^{++} Vit. B_6	4.0	"	842	550
Zn^{++}, Mn^{++}, Fe^{++} Vit. B_1 and B_6	4.2	"	808	554

minerals to the medium had the effect of increasing growth and fibrinolytic activity, while producing a shifting of pH (measured by direct reading on Beckman's) toward the alkaline side. Considerable enhancement of activity was obtained with addition of Zn^{++}; Zn^{++} and Fe^{++}; and Zn^{++}, Fe^{++} and Mn^{++} to the medium. Oddly enough, addition of combinations of Zn^{++} and Mn^{++} or of Fe^{++} and Mn^{++} to the medium did not enhance fibrinolytic activity, although the first combination increased the growth appreciably. The combination of Zn^{++} and Fe^{++} appeared primarily responsible for the increased yield of active substance, which was further enhanced by the addition of Mn^{++}. Addition of vitamins did not appear to be important, since their presence in the medium without trace minerals did not increase growth or activity.

Substitution of NH_4 salts for NO_3 salts to the medium enhanced the growth of the mycelium but did not influence the yield of Aspergillin O. This, in fact, dropped to zero. Thus, although a close relationship usually existed between these 2 variables, abundant growth is not necessarily a criterion for predicting the yield of Aspergillin O by the fungus. It should be noted, however, that the pH was shifted to a very acid value and this change might have destroyed any active material produced. Addition of KNO_2 in lieu of KNO_3 failed to induce growth, production of Aspergillin O or pH changes in the medium. Environmental conditions were relatively insignificant. Exposure of cultures to sunlight inhibited both growth and activity. Culturing at 37°C apparently reduced amount of growth, but did not affect the production of Aspergillin O. Further studies have indicated that *Aspergillus oryzae* (B-1273) seems unable to utilize lactose but will produce material optimally in the presence of either sucrose, maltose, dextrose, galactose or fructose. It has also been observed that the C/N ratio of the medium as used is optimal for growth and production of Aspergillin O. Any increase in carbon (carbohydrate) concentration resulted in decrease in pH and loss of fibrinolytic activity in the filtrates.

Discussion. The results of these studies indicate the possibility of greatly increasing the yield of fibrinolytic material from *Aspergillus oryzae* (B-1273) by addition of trace minerals to the culture medium. Whether the nutritional requirements described here are specific for production of Aspergillin O or are generic requirements for optimal metabolic activity of the fungus is being investigated.

Summary. A method is described for obtaining higher yields of fibrinolytic material (Aspergillin O) from cultures of *Aspergillus oryzae* (B-1273) by addition of trace minerals (zinc, iron and manganese) to the liquid medium, incubated at room temperature and in darkness.

Authors acknowledge assistance of Quartermaster Research and Development Center, Dept. of Army, Natick, Mass. and of Dorothy Fennel for providing parent cultures and their identification. Also acknowledge cooperation of Dr. Hector Marin and technical assistance of Sylvia Karatza in early phase of work. Joanne Macdonald rendered technical assistance.

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Received July 16, 1959. P.S.E.B.M., 1959, v102.

Extent of Total Hydrolysis of Dietary Glycerides during Digestion and Absorption in the Human. (25192)

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The opportunity to obtain thoracic duct lymph from human beings(1,2) has prompted us to undertake a study of the extent of hydrolysis of dietary glycerides during digestion and absorption in man. From animal experiments it is known that glycerol once liberated from the triglycerides by hydrolysis in the intestinal lumen is not utilized for resynthesis of glycerides. The ratio of label in the acid to that in glycerol of the lymph glycerides after feeding double labeled glyceride, when compared with the same ratio in the fed glycerides, gives percentage of glyceride molecules which have been completely hydrolyzed during digestion and absorption process.

Methods. Subjects: Our subjects were 2 patients with diagnosed adenocarcinoma of the left lung. Patient A was a 60-year-old man, weight 56 kg. Patient B was a 56-year-old man, weight 72 kg. In both patients a supraclavicular biopsy of the lymph nodes was performed and a plastic cannula was inserted into the thoracic duct as described earlier(1). In Patient A the cannula was inserted into one of the 2 stems of equal diameter present on operation. In patient B the catheter was inserted into the thoracic duct in such a way that the major portion of lymph flow bypassed the catheter. Only a portion of total lymph flow was therefore obtained during collection periods from these patients. This fact does not, however, influence the results as these are based on the ratio of label in acid and glycerol of the glycerides. *Labelled material.* Linoleic acid-1-C^{14} and glycerol-1-C^{14} (obtained from Radiochemical Centre, Amersham, England) were synthesized into glyceride *via* the acid chlorides. Free fatty acids were removed by passing the reaction mixtures through Amberlite IRA-400 columns and the triglyceride isolated by chromatography on silicic acid(2). Two different batches of glycerides were synthesized. The test meal for patient A was made from 635 mg doubly labelled glyceride homogenized into 310 ml of test meal (Table I). The fatty acid mixture obtained on saponification had a specific activity of 750 cpm and mg. Glycerol had a specific activity of 493 cpm and mg. Three hundred ml of this test meal was fed this patient at 8 a.m. the day after operation. Patient B was fed 300 ml of a test meal made up from 1.520 mg doubly labelled glyceride in 310 ml formula. The fatty acid mixture had a specific activity of 680 cpm/mg and the glycerol part 880 cpm and mg. The test meal was fed at 8 a.m. the day after cannulation. Test meals furnished around 10 g of glycerides each. Lymph was collected in periods shown in the Tables. Food was given again at 11 a.m. and 2 p.m. *Extraction and fractionation of lymph lipides.* Total lipide of the lymph was extracted with 20 volumes of ethanol:ether 3:1. The solvent was evaporated in vacuo, and total lipides were taken up in petroleum ether (b.p. 60-70°) and made to volume. Aliquots were subjected to chromatography on silicic acid to obtain a neutral fat, non-esterified fatty acid fraction and a phospholipide fraction(2). Then, the non-esterified fatty acids were removed from the neutral fat by chromatography on Amberlite IRA-400(2). The neutral fat fraction (glycerides, cholesterol and cholesterol esters) was subjected to chromatography on silicic acid for separation of cholesterol esters and a triglyceride-cholesterol fraction(2). Lipides in aliquots of test meals were extracted in the same way as described for extraction of lymph lipides. Fatty acids from various glyceride fractions of lymph lipides were obtained after saponification at room temperature with 4% KOH in 95% ethanol and petroleum ether, followed by extraction with alkaline 50% ethanol and reextraction of the fatty acids with light petroleum after acidification. Then the glycerol in the saponification liquor was

TABLE I. Composition of Formula Mixture Fed Daily as Sole Source of Nutrients to Patient A. 1500 ml of this formula contains approximately 1900 calories. The patient was fed 300 ml 5 times a day.

Constituents	Wt, g
Triglycerides, sterol-free	210
Milk protein	470
Dextrose	670
Water	5000

oxidized to formaldehyde with periodate and the formaldehyde steam-distilled and precipitated with dimethyldehydroresorcinol(4). The resultant methylene bismethone was filtered, recrystallized and weighed. The different fractions were plated in infinitely thin layers on aluminum planchets for counting in a gas-flow counter. Determinations of specific activity were also performed by the gas-phase counting technic of Glascock* The results of these latter determinations coincided well with those obtained on plating.

Results summarized in Tables I and II, show that relative specific activities of the fatty acid part of the lymph triglycerides are greater than those of the glycerol part. As the fatty acids liberated by hydrolysis appear in lymph mainly as triglycerides, but liberated glycerol is not reutilized for glyceride synthesis, the lower relative specific activity in the glycerol part is a measure of amount of glyceride, which has been completely split during digestion and absorption. The figures obtained show that when dietary glycerides reach the thoracic duct lymph in man ⅕ to ½ have been completely hydrolyzed. Inside this range, extent of hydrolysis varies considerably with a tendency to lower hydrolysis during early period of digestion and absorption. The figures obtained include loss of glycerol taking place in lumen of intestine and in the mucosa cells. The correspondence of the figures obtained in our experiments and those obtained from studies of intestinal content(5) indicate that no appreciable loss of glyceride glycerol occurs in the cells. The absorbed glycerides must have had one or more fatty acids attached to the glycerol but the figures do not give any indication on the extent of hydrolysis of the glycerides absorbed as such(6). The results of this investigation on extent of total hydrolysis of glycerides during digestion and absorption are well in accord with those calculated for extent of hy-

TABLE II. Analysis of Thoracic Duct Lymph Lipides of Subject A after Ingestion of a Doubly Labeled Triglyceride. Form of fat administered: Linoleic acid-1-C^{14} as triglyceride. Spec. act. of TG-FA = 750 cpm/mg. Glycerol-1-C^{14} as triglyceride. Spec. act. of TG-glycerol = 493 cpm/mg. Glycerol counted as methylene bismethone. Lymph triglycerides were isolated after chromatography on silicic acid.

Collection periods after admin., hr	Lymph vol, ml	Total fat, mg	Lymph glycerides Fatty acid (Relative spec. act.)	Lymph glycerides Glycerol (Relative spec. act.)	% total hydrolysis
1–2	21	577	44.5	30.5	32
3–4	12	218	20.5	16.2	22
4–5	22	772	38.0	31.0	19
5–6	35	2228	55.5	32.2	42
7–8	12	551	16.4	12.4	25

TABLE III. Analysis of Thoracic Duct Lymph Lipides of Subject B after Feeding a Doubly Labeled Triglyceride. Form of fat administered: Linoleic acid-1-C^{14} as triglyceride. Spec. act. of TG-FA = 680 cpm/mg. Glycerol-1-C^{14} as triglyceride. Spec. act. of TG-glycerol = 800 cpm/mg. Glycerol counted as methylene bismethone.

Collection periods after admin., hr	Lymph vol, ml	Total fat, mg	Lymph glycerides Fatty acid (Relative spec. act.)	Lymph glycerides Glycerol (Relative spec. act.)	% total hydrolysis
0–2	39	959	13.2	10.4	21.5
2–3	7.5	473	12.9	6.7	48
3–4	21	381	5.9	3.7	37.5

* These determinations were performed by Dr. S. Lindstedt.

drolysis from thoracic duct studies in the rat (7,8).

The technical assistance by J. Gürtler is greatly acknowledged.

This work is part of investigations supported by Swedish Medical Research Council.

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Received July 16, 1959. P.S.E.B.M., 1959, v102.

Comparative Toxicity of 3-Nitro 4 Hydroxyphenylarsonic Acid and Its Formaldehyde Dimer to Chicks. (25193)

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The arsenical, 3-nitro 4 hydroxyphenylarsonic acid (3-nitro), is widely used in poultry and swine rations. In the feed or drinking water of poultry, 3-nitro has a growth stimulating and coccidiostatic effect(1). In swine feeds, 3-nitro is used for growth promotion and as an aid in controlling infectious enteritis(2). Toxicosis has been reported in both species when 3-nitro was fed at recommended levels for growth stimulation or disease control(3,4). Polybenzarsol, a mixture of polymers formed from the reaction of formaldehyde and 4-hydroxy benzenearsonic acid, has been reported to have a low order of toxicity in rats, mice, dogs and man, following oral administration(5). This report suggests the possibility that the reaction product of formaldehyde and 3-nitro 4 hydroxyphenylarsonic acid (hereafter called the dimer) might have reduced toxicity to poultry.

Methods. The dimer of formaldehyde and 3-nitro was synthesized using the method of Faith(6). This was fed to chicks in 2 experiments. The basal diet was a practical broiler feed deemed adequate in all known nutrients. The birds were housed in electrically heated batteries equipped with raised wire floors and allowed access to feed and water *ad lib.* In the first experiment, duplicate lots of 10 male broiler strain chicks were given the following treatments: control; 3-nitro at levels of 49.5 and 198.0 mg/kg of feed; the dimer of 3-nitro at levels of 51.15 and 204.6 mg/kg; and procaine penicillin at a level of 4.4 mg/kg. All treatments in the second experiment were triplicated. Each lot contained 5 male and 5 female chicks. Treatments were as follows: control; 3-nitro at levels of 49.5, 74.25, and 198.0 mg/kg of diet and the dimer of 3-nitro at levels of 51.15, 76.72, and 204.6 mg/kg. At end of the 4-week test, triplicate liver arsenic determinations were made for each level of arsonic fed.* In both experiments all chicks were wing banded and individual weights taken at initiation and at termination of test. Feed consumption was determined by group at weekly intervals. Acute toxicity was studied using 4-week-old male chicks given a single oral dose of 3-nitro at levels of 100 and 200 mg/kg body weight and the dimer at levels of 103.2 and 206.4 mg/kg. The technic has been described(7).

Results. Data obtained in Exp. 1 and 2 are shown in Table I. In the first experiment, 4.4 mg penicillin, 49.50 mg 3-nitro and 51.15 mg of the dimer/kg diet respectively significantly improved growth over that obtained on the basal diet ($P = 5\%$ or less). 3-nitro appeared to be more effective in stimulating growth than either penicillin or the dimer, the latter 2 being essentially identical in their

* Arsenic determinations were made by J. B. Thompson of Trace Metal Research Labs., Chicago Heights, Ill.

TABLE I. Effect of 3-Nitro and Its Dimer on Chick Growth, Feed Efficiency and Liver Storage of Arsenic.

Arsenic addition (mg/kg)		Avg wt gain (g) 0-4 wk		Feed efficiency*		PPM As_2O_3
3-nitro	Dimer	Exp. 1†	Exp. 2‡	Exp. 1	Exp. 2	in liver
		317	305	1.87	1.78	.17‖
49.50		390	346	1.67	1.81	.86
	51.15	376	328	1.77	1.72	1.60
74.25			352		§	1.14
	76.72		375		§	2.0
198.00		284	293	1.78	1.79	2.0
	204.60	307	289	1.79	1.67	2.9
(4.4 procaine penicillin)		361		1.89		

* g feed/g gain. † Avg of replicated lots. ‡ Avg male and avg female/2; avg of triplicated lots. § 4th wk feed consumption data misplaced. ‖ Avg of 3 assays—each sample consisting of composited whole liver from 1 ♂ and 1 ♀ chick.

effect; however, the differences were not statistically significant. At the level of 198.0 mg/kg, 3-nitro significantly depressed growth ($P = 5\%$) whereas 204.6 mg/kg of its dimer, compared with the controls, was without significant effect.

In Exp. 2, growth was significantly improved ($P < .01 > .001$) by all additions except the highest level of 3-nitro and its dimer and the 51.15 mg level of the dimer. The difference between growth on the 76.72 mg/kg level of the dimer and the 49.5 mg/kg level of 3-nitro approached significance ($P > .05$). Growth was not significantly depressed by the highest level of arsonics. At each level of comparison, the dimer resulted in greater liver stores of arsenic than did 3-nitro (Table I).

As shown in Table II, there was essentially no difference in toxicity, expressed as number and weight change of survivors, between a single dose of 100 or 200 mg 3-nitro/kg body weight and an equivalent dose of the dimer. All treated birds lost weight. And, although the birds given the dimer appeared to be less affected, there were no statistically significant differences.

Discussion. The data obtained in both feeding trials suggest that at a level comparable to 49.5 mg of 3-nitro/kg of diet, the dimer of 3-nitro is slightly less growth stimulatory. This level corresponds to the commercial usage of 3-nitro, i.e., 45 g/ton. At an intermediate level, the dimer produced greater (but non-significant) weight gains. In one experiment in which growth was depressed by highest levels, the dimer was less detrimental.

TABLE II. Toxic Effect of a Single Oral Dose of 3-Nitro and Its Dimer.

Treatment	Avg change in body wt (g), 3 days	
Control	+64.7	(10)*
100 mg 3-nitro/kg	−40.7	(7)
103.2 " dimer/kg	−36.6	(8)
200 " 2-nitro/kg	−38.0	(1)
206.4 " dimer/kg		(0)

* No. surviving of 10 chicks started.

Wharton *et al.* reported the LD_{35} dose of 3-nitro, administered/*os* to chicks 4 weeks of age, was 100 mg/kg of body weight(7). Weight loss of survivors averaged 48 g. This is in good agreement with LD_{30} and 40 g weight loss obtained in this study with the 100 mg/kg level of 3-nitro. The dimer, with an apparent LD_{20} and 36 g weight loss at a comparable dose, is little, if any, less toxic. 200 mg of 3-nitro/kg body weight is essentially the LD_{100} dose for chicks(7). Our data show that LD_{100} of the dimer is no greater than 200 mg/kg.

The consistently higher liver arsenic stores in chicks receiving the dimer suggest that it is excreted at a slower rate than 3-nitro.

Toxicity to chicks of 3-nitro was not greatly different from that of its formaldehyde dimer. This is in contrast to the report that the formaldehyde polymer of 4 hydroxy benzenearsonic acid has greatly reduced toxicity to rats, mice and dogs. Rats tolerated the polymer at levels of 400 mg/kg/day for 8 weeks and there was no mortality in mice given 4 g/kg intragastrically as a single dose(5).

Summary. The formaldehyde dimer of 3-nitro 4 hydroxyphenylarsonic acid was slightly less growth stimulatory than 3-nitro when fed to chicks at a level equivalent to 49.5 mg of 3-nitro/kg diet. Both arsenicals

stimulated growth when fed at a level equivalent to 74.25 mg of 3-nitro/kg diet, with the dimer somewhat more effective. There was little difference between the 2 when fed at a level equivalent to 198 mg 3-nitro/kg diet. Liver arsenic stores were greater in chicks fed the dimer at each of the 3 levels of comparison. Administered as a single oral dose at levels approximating 100 and 200 mg/kg body weight, survival out of 10 chicks treated was 7 and 1 respectively, for 3-nitro and 8 and 0 for the dimer.

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Received July 17, 1959. P.S.E.B.M., 1959, v102.

Hypoglycemic Activity of Tris Buffer in Man and Dog. (25194)

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Tris(hydroxymethyl)aminomethane [$(CH_2OH)_3 \cdot C\text{-}NH_2$; tris buffer, THAM] has been used widely in biochemical work as a buffer. Nahas(1) discovered that it can be safely infused into dogs and corrects profound respiratory acidosis. Stimulated by this observation, our laboratory participated in a study of the alkalinizing effects of intravenous tris in healthy, fasting adult man. Since little was known of *in vivo* properties of tris, we set out to characterize its effects in terms of chemical measurements available in our laboratory. We were surprised to find a hypoglycemic effect.

Studies in man. After an oral water load, 4 subjects breathed room air for about half an hour and then 2.3 or 3.4% CO_2 for a similar period. Intravenous tris (0.3M in 0.03M NaCl and 0.005M KCl) was given immediately thereafter, during CO_2 breathing, over 30 to 64 minutes. A recovery period followed. Arterial blood was sampled from an indwelling femoral cannula. Serum glucose(2) fell distinctly (Table I) in 3 subjects during and at the end of tris infusion. Hypoglycemia occurred in J.F., who received the largest dose (8.8 mM/kg or 0.49 ml/kg/min over 60 minutes). This was accompanied by hunger and profuse sweating superseded by other toxic manifestations which persisted for 24 hours. Glucose returned toward pre-infusion values within 57 minutes after infusion in J.F. Glucose fell substantially in S.B., 2.8 mM tris/kg (0.19 ml/kg/min over 49 minutes) and in J.D., 4.1 mM/kg (0.21 ml/kg/min over 64 minutes). The fall in J.L. to 65 mg/100 ml occurred 26 minutes after tris infusion was over (3.0 mM/kg or 0.33 ml/kg/min for 30 minutes). Serum inorganic phosphate fell progressively with glucose in J.F. from 3.6 to 1.9 mg/100 ml, from 3.5 to 2.9 mg/100 ml in J.D., from 3.8 to 3.3 mg/100 ml in J.L., but was unchanged in S.B. Serum potassium did not fall in any of the subjects. Other chemical and physiologic observations are reported elsewhere(3,4).

Studies in dogs. Tris was then given intravenously to 3 fasting, anesthetized (pentobarbital), heparinized, female dogs during room air breathing. 0.3M saline was infused at 0.5 ml/kg/minute over 1 hour; then 0.3M tris (in 0.03M NaCl and 0.005M KCl) at the same rate (slow infusion, Table II) for 2½ to 3 hours followed by 1 hour at 0.7 to 1.0 ml/kg/minute (fast infusion, Table II). Femoral arterial serum glucose concentrations rose during anesthesia and manipulation of 2 of the animals and then tended to remain unchanged or return to pre-anesthetic levels during saline infusion. Glucose declined during slow infusion of tris in all dogs. Values fell to below 50 mg/100 ml in 2 dogs. Rapid infusion produced a precipitous further reduc-

tion in serum glucose in all dogs. Serum inorganic phosphate fell to less than 50% of control values in all dogs, during tris injection, reaching levels consistently below 1.0 mg/100 ml in 2 of the animals. This fall probably signified a net transfer of phosphate from extracellular fluid to cells, since urinary phosphate excretion decreased. There was no definite trend in serum potassium.

Other findings related to tris administration in the dogs include: Blood pH rose by 0.24 to 0.30 units and plasma CO_2 content increased by 4 to 9 mM/L. Serum sodium fell by 14 to 27 and chloride by 17 mM/L. Arterial blood pressure rose, and remained high, after the first 2 to 2½ hours of tris. Urine flow and excretion of sodium, chloride, and potassium rose.

Glycosuria did not develop during tris injection into either man or dog. *In vitro* additions of tris greatly exceeding estimated concentrations *in vivo* did not artifactually lower glucose or phosphate.

Discussion. The present experiments do not establish whether the fall in serum glucose induced by tris is mediated by insulin, is a specific hypoglycemic effect independent of insulin, or represents diminished gluconeogenesis. Work is in progress to examine these possibilities. The phosphate fall resembles that following glucose and/or insulin administration. Lack of fall in serum potassium may reflect the small load of potassium given with tris rather than a difference from the potassium-lowering tendency of glucose and insulin.

TABLE I. Arterial Serum Glucose in 4 Adult Males. (No. in parentheses represent min. from start of tris or recovery period.)

Glucose, mg/100 ml			
S.B. 88.5 kg	J.D. 83.2 kg	J.F. 71.7 kg	J.L. 75.5 kg
	Room air		
91	102	100	75
	CO_2		
97	111	89	80
	Tris and CO_2		
78 (30′)	90 (32′)	55 (39′)	77 (18′)
74 (49′)	80 (64′)	52 (56′)	76 (25′)
	Recovery		
75 (60′)	86 (98′)	79 (57′)	72 (5′)
		89 (122′)	65 (26′)

TABLE II. Arterial Serum Glucose in 3 Dogs.

	Glucose, mg/100 ml		
Time, min.	#110 14.7 kg	#111 22.2 kg	#112 13.2 kg
	Pre-anesthesia		
		92	114
	Pre-NaCl (2-3 hr later)		
	100	131	122
	NaCl infusion		
30	94		114
60	93	128	106
	Tris infusion (slow)		
30	84	110	103
60	73	94	84
90	77	85	88
120	53	69	78
140	45*	53	79
160		37	79
180		30	78
	Tris infusion (fast)		
15		26	60
30	13	25	60
45		10	43

* Slow infusion ends.

The hypoglycemic action of tris in the intact organism has broad implications. Clinically, it emphasizes one of the possible sources of toxic side effects if the amine is used for treating acidosis. Nevertheless, it is conceivable that this or related compounds may prove useful in treating diabetes mellitus or diabetic acidosis. Moreover, studies of the relation of tris and similar compounds to carbohydrate metabolism both *in vivo* and *in vitro* may provide a fresh approach to problems of carbohydrate regulation.

Summary. Tris(hydroxymethyl)aminomethane produced significant hypoglycemia in man and dog. The nature of this phenomenon remains to be determined.

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Received July 20, 1959. P.S.E.B.M., 1959, v102.

Mycoplasmal (PPLO) Polyarthritis and Tumor Regression in Rats.*† (25195)

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Mycoplasma arthritidis, a pleuropneumonia-like organism (PPLO), has been reported to be a causal agent of polyarthritis in rats. Woglom and Warren encountered a filterable agent from abscesses occurring at the site where rat sarcoma had been transplanted(1). When this material was injected intravenously into normal rats, most of the surviving animals developed polyarthritis. Klieneberger (2) and Woglom and Warren(3) subsequently demonstrated the filterable agent to be a PPLO. Findlay, *et al.*(4) reported isolation of PPLO from affected joints of rats with spontaneous polyarthritis. Arthritis was produced by injecting sterile agar and a filtrate of the ground infected joints or a rabbit blood culture of PPLO into the pad of the hind foot of normal rats. Similarly, polyarthritis in normal rats has been produced by intracardiac injection of large volumes of an ascitic fluid broth culture of PPLO of the L4 type(5) or by intraperitoneal injection of broth cultures of PPLO(6). Jasmin(7) produced a proliferative arthritis in rats by intraperitoneal or intravenous injection of exudate collected from an air pouch containing lymphosarcoma. When this exudate was cultured in chick embryos a pleomorphic organism was isolated which, when subcultured on solid media, was similar to the L4 strain of PPLO. The sporadic occurrence of polyarthritis in rats during regressing of Murphy-Sturm lymphosarcoma induced by *Klebsiella pneumoniae* polysaccharide has been observed in our laboratory. Cultural studies yielded growth of PPLO, *Mycoplasma arthritidis*, from the involved periarticular tissues and the necrotic tumor. The present study was undertaken to investigate the respective roles of the PPLO, the tumor necrotizing bacterial polysaccharide, and the tumor regression in production of the polyarthritic lesions.

Material and methods. The Murphy-Sturm lymphosarcoma was propagated in male Sprague-Dawley rats weighing 90 to 110 g. Tumor tissue was aseptically minced, suspended in Earle's solution, and injected subcutaneously into the lumbo-spinal region. A palpable tumor appeared at site of injection in 6 to 10 days and grew rapidly to a spheroid mass of about 60 g measuring approximately 50 × 70 mm before death of the rat. Occasionally the tumor regressed spontaneously before reaching a diameter of 30 × 35 mm. Necrosis of the tumors was produced by 4 daily intraveneous injections of *Klebsiella pneumoniae* polysaccharide, 0.1 mg/100 g body weight. If the tumors were less than 35 mm in diameter, disappearance of the tumors usually resulted. Preparation of the polysaccharide has been described(8). PPLO were isolated and transplanted on modified Shepard's media(9) using Fields' tryptic digest broth (B-B-L). Colonies were examined by the stained cover-slip agar block method of Dienes(10). Difco PPLO broth, supplemented with phenol red dextrose broth and PPLO serum fraction, was used for fermentation studies. "PPLO-free" tumor-bearing rats were obtained by subcutaneous injections of 3.3 mg of tetracycline HCl twice daily for 7 to 10 days, beginning on the day of or the day before tumor transplant. These rats showed no arthritic lesions and their tumors were culturally negative for PPLO. On the 8th or 9th day following tumor transplant, 0.5 ml of a 4 day broth culture of PPLO was injected by tail vein. The first injection of polysaccharide was on the 10th or 11th day after tumor transplant. To evaluate the interrelationship of the 3 variables—PPLO, bacterial polysaccharide, and tumor regression —9 groups of male rats of similar age were used (Table I). Groups 1 through 4 were tumor-bearing rats. Of these, groups 1 and 2 were "PPLO-free," and 3 and 4 were PPLO-

* Supported, in part, by research grants from Nat. Inst. Health, U.S.P.H.S.

† Presented, in part, before Intermountain Branch, Soc. Am. Bact., Pocatello, Idaho, May 1959.

TABLE I. Rat Tumor Regression and PPLO-Arthritis.

	Tumor bearing rats				Non-tumor controls		Prior (spontaneous) tumor regression
	PPLO-free		PPLO-inj.		PPLO-free	PPLO-inj.	PPLO-inj.
	Tumor regression						
	Pos.	Neg.	Pos.	Neg.			
	(Group 1)		(Group 3)		(Group 5)	(Group 7)	
Polysaccharide	*0/9	0/3	6/7	6/9	0/10	0/16	
	(Group 2)		(Group 4)		(Group 6)	(Group 8)	(Group 9)
No polysaccharide	0/1	0/9	4/4	4/11	0/10	0/10	9/9

* Numerator = No. of rats with arthritis. Denominator = No. of rats in group.

injected. Groups 1 and 3 received polysaccharide. Groups 5 through 8 were non-tumor control animals treated as in Groups 1 through 4. In Group 7, PPLO were injected into 10 rats 3 days prior to polysaccharide injection, into 3 rats on the third polysaccharide injection day, and into 3 additional rats on the day following the fourth polysaccharide injection. In addition, 9 rats (Group 9) with spontaneously regressed tumors and "PPLO-free" were injected with PPLO on the 5th to 8th day after onset of tumor regression.

Results. Twelve "PPLO-free" tumor bearing rats were injected with polysaccharide (Table I, Group 1). The tumors of 9 regressed; the other 3 showed only temporary inhibition of tumor growth. No arthritis was observed in any of the 12 animals. Similarly, arthritis did not develop in the 10 "PPLO-free" tumor-bearing rats which received no polysaccharide. Spontaneous tumor regression occurred in one of these animals (Table I, Group 2).

In the PPLO-injected animals, Groups 3 and 4, tumor regression was likewise more frequent following injections of polysaccharide. In Group 3, tumor regression occurred in 7 of 16 rats. In Group 4, the tumors of 4 of the 15 rats regressed spontaneously. In Groups 3 and 4, arthritis was seen in all the rats with tumor regression except one. In Group 3, nine rats had only temporary inhibition of tumor growth (negative tumor regression); 6 had arthritic lesions with extensive areas of necrosis in the tumor.

In the non-tumor control groups, no arthritis was observed in the PPLO-free or PPLO-injected groups with or without polysaccharide (Table I, Groups 5, 6, 7, 8). In contrast, 9 "PPLO-free" tumor-bearing rats with spontaneous tumor regression developed polyarthritis within 2 to 4 days following injection of PPLO (Table I, Group 9).

PPLO were recovered from all arthritic joints cultured; likewise, cultures from tumors of PPLO-injected rats yielded PPLO growth.

FIG. 1. Periarthritis in hind foot of rat with prior regressed lymphosarcoma. Arthritis developed 4 days after intravenous injection of *Mycoplasma arthritidis* (PPLO).

No PPLO were recovered from joints or tumors of "PPLO-free" tumor-bearing rats or from joints or tissue of non-tumor control rats.

Comments. Our data indicate that the significant condition for production of PPLO arthritis in rats was the concurrent or prior necrosis of Murphy-Sturm lymphosarcoma. Since polyarthritic lesions resulted from PPLO infection of tumor regressed rats, neither the bacterial polysaccharide, viable tumor nor actual necrotic tumor appears essential for induction of arthritis.

Production of acute joint lesions by bacterial polysaccharide in normal guinea pigs (11) suggested a similar role in the arthritis of tumor-bearing rats. However, in PPLO-injected rats without tumors, injection of bacterial polysaccharide resulted in no arthritis. That PPLO might localize in the articular structures only during a transient favorable phase following polysaccharide injection seems untenable since the polysaccharide was introduced at various intervals before and after PPLO injection. Likewise PPLO-infected arthritic lesions were observed in tumor-regressed rats that received no polysaccharide.

Although the arthritis occurred occasionally in some rats with progressively enlarging lymphosarcoma, areas of necrosis were found within such tumors. It should be emphasized that arthritis was much more frequent in the rats with regressing than with progressing tumors. Since in the PPLO infected animals the best source of the microorganisms was from tumor tissue, it may be proposed that the necrosis of the tumor leads to the massive release of PPLO into the blood stream with subsequent localization in articular or periarticular tissues. However, release of PPLO from necrotic tumor tissue would not appear essential inasmuch as the non-infected rats with complete, though recent, spontaneous regression of tumor developed arthritis within 2 or 3 days after intravenous injection of PPLO.

Various studies suggest that PPLO arthritis in rats may occur with certain tissue reactions other than tumor necrosis. Necrotizing agents such as croton oil(7), chronic inflammatory agents such as Freund's adjuvant(12), or tumor in an air pouch(7) may have a common denominator of host response to the necrotic debris as the essential preparatory mechanism for PPLO arthritis. The nature of the significant components of necrotic tissue, the cellular and humoral aspects of host response and the favorable conditions for PPLO localization and growth in articular structures are unknown.

Summary. Sporadic polyarthritis in rats observed during spontaneous and induced regression of a lymphosarcoma has been investigated. Cultural studies of the involved periarticular tissues and the tumor yielded PPLO, *Mycoplasma arthritidis*. The respective roles of PPLO, tumor-necrotizing bacterial polysaccharide, and tumor regression in production of the polyarthritic lesions have been evaluated. Arthritis occurred only in PPLO-infected rats with concurrent or prior necrosis of tumor tissue. Bacterial polysaccharide and viable tumor were not essential.

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Received July 20, 1959. P.S.E.B.M., 1959, v102.

Macromolecular Growth Requirements of Human Cells in Continuous Culture.* (25196)

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It is now well established that human cells in continuous culture provide valuable tools in studies of metabolism and genetics. There remain, however, 2 important limitations which frequently give rise to conflicting results(1,2); the necessity of incorporating serum proteins in assay media and the unpredictable evolution of cell populations. It is well known that dialyzed serum contains numerous components, active biologically in trace quantity such as enzymes, hormones, vitamins and trace metals. It is not surprising that experimental results may be affected by the source of sera(2,3). To eliminate the variable introduced through serum proteins, investigations have been directed chiefly towards development of chemically defined media. Indeed, preliminary reports indicate that variants of human epithelial-like cells derived from skin have been successfully adapted to propagate in chemically defined media(4). While this development undoubtedly represents an important advance in tissue culture methodology, the importance of the reported existence of intracellular proteins immunologically related to various serum proteins should not be minimized(5). With this view in mind, the staffs of the Dept. of Microbiology, Harvard University and the Protein Foundation have jointly undertaken a long term study to elucidate the roles of various serum macromolecules in the physiology of human cells in continuous culture. This report describes the isolation of serum fractions capable of promoting and inhibiting cell growth. While there are reports on the isolation of alpha globulin-like substances capable of enhancing cell attachment to glass surfaces(6,7), studies on promotion of prolonged cell growth are lacking. To the best of our knowledge, there also has been no report of serum fractions which when diluted in a complete growth medium, cause cellular degeneration.

Materials and methods. Preparation of serum and plasma fractions. Serums were collected and stored as described previously(8). Plasmas were prepared both from blood collected by standard procedures (in ACD anticoagulant solution) and from blood collected through resin columns and separated in the Cohn-ADL blood fractionator(9). Plasma was always held refrigerated until fractionated, and after the lability of the growth promoting factors had been noted, it was processed immediately after collection or was frozen and held in the frozen state until fractionated. Human serums and plasmas were fractionated in pools of 3 to 10 donors while equine serums were fractionated individually. Cold ethanol method 6(10) was used throughout this study. Zinc method 12(11) was equally promising in initial studies but was abandoned because of the more extensive knowledge of the subfractionation of the cold ethanol fractions. Both methods were designed for human plasma. To obtain more reproducible results, we have found it desirable to complete the fractionation procedure within 7 days and to remove the ethanol from the fractions by dialysis at 0°C against 2 changes of 2 liters each of 0.85% NaCl for 24 hours instead of by lyophilization. The fractions were prepared in concentrated form and were kept frozen at below –60°C. *Human cells.* The HeLa(12) and the conjunctival cells(13) derived respectively from malignant and normal human tissues were used. Stock cultures were nourished in the minimal growth medium containing 10% dialyzed horse serum described by Eagle(14) and modified in this laboratory by omission of biotin, addition of meso-inositol to 10 μM and substitution of Earle's balanced salt solution by Hank's. These cultures have been examined at regular intervals and found free of con-

* Supported by research grants from Public Health Service.

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TABLE I. Propagation of Conjunctival Cells in Fractions from a Pool of Human Serums.

Fraction	Concentration S.E.,* %	Concentration Protein†	No. fold increase per weekly subculture 1st	2nd	3rd
II + III	50	.93	<2	deg.§	
"	10	.19	<2	"	
IV-1	50	.24	deg.		
"	10	.05	"		
IV-4	50	.22	5	12	16
"	10	.04	4	2	deg.
V	50	1.52	2	deg.	
V	10	.30	deg.		
Combined‡	50	3.29	5	8	3
"	10	.66	6	4	deg.
Serum‖		.66	20	18	14

* S.E. = Serum equivalent.
† Protein concentration in g/100 ml media as calculated from values reported by Cohn, *et al.*(10).
‡ Combination of fractions II + III, IV-1, IV-4, and V.
§ Complete degeneration.
‖ Serum from same pool without fractionation but dialyzed; used at dilution of 1/10.

tamination by pleuropneumonia-like organisms. Methods used in assaying cell multiplication have also been described(8). *RNA and RNAse determination.* Ribonucleic acid was determined by incubating 2 ml of serum fraction with 10 μg of crystalline ribonuclease at pH 5 for 24 hours at 37°C. The mixture was dialyzed against an equal volume of water at 0°C for 48 hours and the dialyzing fluid was assayed for ribosides by the orcinol reaction as well as by UV light absorption at 260 mμ. Ribonuclease activity was tested by incubating approximately 1 mg of yeast RNA with 2 ml serum fraction at pH 7.6 for 48 hours at 37°C; the amount of dialyzable ribosides was then assayed as described for determination of RNA. These conditions were selected to mimic those of cell cultivation. *Vitamin assays.* Total niacinamide and B_{12} content of various fractions were measured by the standard microbiological technic(15).

Results. Assays of growth promoting activity. The results obtained with fractions from a pool of human serum are presented in Table I; growth promoting activity is associated chiefly with fraction IV-4. It should be emphasized that while some growth promoting activity has been consistently detected in most fractions IV-4, the results obtained with different pools of human serum or plasma show important differences. There has been a tendency for less active fraction IV-4 to be obtained from pools in which the cytotoxic activity described below is most pronounced. Two out of 18 lots of fraction IV-4 has been found completely inactive at 50% serum equivalent concentration, and several lots of IV-4 were incapable of sustaining growth for more than one subculture. Recombination of fractions, even when an active fraction IV-4 has been obtained, frequently failed to sustain growth. Addition of serum ultrafiltrate to a final concentration of 10% generally improved and prolonged cell growth but did not alter qualitatively the results of such experiments. Subfractions of fraction IV-4(16) have failed to show the growth promoting activity of the parent fraction. Three pools of human serums were also fractionated by zinc method 12,(11); growth promoting activity was detected in zinc insoluble fractions (PGP). Contrary to human sera, growth promoting activity of equine serum was found to be associated chiefly with fraction II + III as illustrated in Table II. Two individual equine sera have been studied thus far with similar results. No significant qualitative difference has been observed thus far between the conjunctival and HeLa cells. In consideration of these results it must be remembered that a fractionating system designed for human plasma has been applied to equine serum

TABLE II. Multiplication of Conjunctival Cells in Fractions from An Equine Serum.

Fraction	Conc., %*	Fold increase/weekly subculture 1st	2nd	3rd
II + III	50	17	20	16
	10	4	deg.†	
IV-1	50	10	"	
	10	2	"	
IV-4	50	10	"	
	10	deg.		
V	50	2	"	
	10	deg.		
Combined‡	50	12	10	8
	10	12	5	9
Serum§		13	18	11

* % serum equivalent concentration.
† Complete cellular degeneration.
‡ Combination of all fractions.
§ Unfractionated but dialyzed; tested at 1/10 dilution.

and that little is known about the distribution of proteins in the fractions so obtained, and, indeed it is unlikely that the distribution would be the same as that obtained from application of the system to human plasma or serum.

Assays for growth inhibitory activity. Our observation that combination of all human serum fractions frequently were incapable of sustaining cell growth suggested that certain fractions may be growth-inhibiting or cytotoxic. Screening for cytotoxic activity was performed by assaying cell growth in the modified Eagle's basal medium containing 10% horse or human serum with the further addition of test fractions. Results of several screenings consistently indicated that cytotoxic activity was associated with human fraction II + III. No toxic fraction has yet been found with equine sera. Quantitative study of cytotoxic activity of fraction II + III for the HeLa cell is presented in Table III. Of interest is the fact that human serum pool C consisted of sera from 4 selected donors whose sera had been pretested, found satisfactory and used regularly during the past 4 years for cell cultivation. Fraction II + III from this pool was interestingly of low cytotoxicity. Similar results were obtained for the conjunctival cell. In one single experiment, fraction II + III from pools A, B, and D were markedly cytotoxic for primary explant of human amnion cells. Two lots of human fraction II + III were further subfractionated by the method of Oncley, *et al.*(17). In both instances, cytotoxicity was present in subfraction II + IIIw. When II + IIIw was further subdivided into fractions II-1,2,3 and III, neither of these was cytotoxic. When II-1, 2,3 and III were recombined, however, cytotoxicity was again demonstrable. Of interest is the absence of demonstrable cytotoxicity in subfractions III-0, III and II-1,2,3 in which lipoprotein, proteolytic enzymes and gamma globulins are concentrated respectively. To exclude the possibility that cytotoxicity of fraction II + III may have been artificially introduced through fractionation, cell propagation in 4 human serums were tested undiluted and at 10% concentration; in all instances, advanced degeneration were noted in undiluted serums in contrast to the 10-25 fold increases in 10% serum.

TABLE III. Propagation of HeLa Cells in Minimal Growth Medium with the Addition of Fraction II + III.

Serum pool	Conc. II + III tested, %*	No. cell × 10^3/culture 0 day	7th
Human A	100	16	0, 0†
	25	"	88, 70
B	100	"	0, 0
	25	"	6, 14
C	100	"	238, 272
	25	"	311, 249
D	100	"	0, 0
	25	"	90, 62
Control	0	"	290, 274
Equine 1	100	24	456, 474
2	100	"	552, 638
Control	0	"	276, 374

* Conc. fraction II + III diluted in modified Eagle's basal medium containing 10% dialyzed human or horse serum; conc. expressed as percent of equivalent conc. in unfractionated serum.

† Results of replicate culture.

Vitamins, RNA and RNAse contents of growth promoting fractions. Since some tissue culture investigators tend to consider serum fractions as homogeneous macromolecules, the presence or absence of molecular species (Vitamin B_{12} and Niacin), non-protein macromolecules (RNA) and enzymes of low molecular weight (RNAse) was determined. Table IV tabulates such results which are self-explanatory.

Discussion. Bazely and his associates, assaying serum fractions with primary explants of monkey kidney cell by a crude short term method, reported growth promoting activity in fractions IV and V of human, equine and bovine serums(18). Sanford and her associates found growth promoting activity for the L strain of mouse fibroblast in all fractions (19). Since the cells assayed by these workers were capable of surviving or slowly multiplying in the chemically defined medium employed as diluent for the duration of their experiments, the activity measured may have been related to stimulation of growth (as determined by net increase in cell number).

More closely related to the present study are the reports of Lieberman and Ove(6), Fisher, Puck and Sato(7) and Chang and

TABLE IV. B_{12}, Niacin, RNA and RNAse Contents of Human Fraction IV-4 and Equine Fraction II + III.*

Serum	Fraction	B_{12}, mμg	Niacin, μg	RNA (μg) Orcinal	RNA (μg) UV	RNAse
Human #1	Unfractionated†	.2	.02	3.4	2.6	+
	IV-4	.01	.01	0	0	+
#2	Unfractionated	.5			3	+
	IV-4	.01	.02	0	0	+
#3	IV-4	<.01	.04	0	0	+
Equine #1	Unfractionated	2.9	.07		2	+
	II + III	.13	.03	0	0	+
#2	Unfractionated	1.2	.05			+
	II + III	.17	.04	0	0	+

* Expressed as amt/ml; fractions were diluted to equivalent concentrations in sera.
† All unfractionated sera were extensively dialyzed.

Geyer(8). Lieberman and Fisher reported that an alpha-globulin fraction actively promoted cell attachment to glass and that cell multiplication would occur for the short duration of their experiments if autoclaved peptone or albumin were further added. Chang and Geyer found that fractions soluble in 17.5% Na_2SO_4 were capable of sustaining cell growth for at least 2 months. More recently, Lieberman reported complete loss of activity upon purification of fetuin, an alpha-globulin which has been suggested to be active in promotion of the growth of tissue cells(20).

Our results showed that the essential growth factors of serum proteins were concentrated chiefly in fraction IV-4 (46% alpha-globulin, 38% beta-globulin and 16% albumin) of human serum. When zinc method 12 was employed, however, the growth promoting activity accompanied the zinc precipitable proteins (PGP) which are, in general, dissimilar to the proteins of fraction IV-4. The alpha-globulins are generally not zinc precipitable. The presence of growth sustaining activity in horse serum fraction II + III rather than IV-4 indicated possible differences in the physicochemical properties of these factors between those of human and equine origin. Frequent variations in the growth promoting activity of fraction IV-4 from different lots of human serum served to reiterate the importance of testing several lots of serum in the study of cell physiology to minimize conflicting results.

The presence of cytotoxic fraction in most human serums tested deserved comment. This finding may explain the fact that cell cultures often do better with serum at 10-25% rather than 50-100% concentrations; it also suggests that toxicity of some serums is inherent in the serum rather than introduced through faulty technic. Of interest is the absence of cytotoxicity in fraction III-0 (rich in lipids), fraction II-1,2,3 (rich in gamma globulins) and in fraction III (rich in proteolytic enzymes); these factors have been considered by some investigators as deleterious to *in vitro* cell growth. The possibility that the cytotoxic factors may act as a barrier to hematogenous migration of somatic cells and as growth inhibitor *in vivo* deserves further exploration.

The co-existence of growth promoting and inhibiting factors in varying concentrations substantiates an earlier observation of the desirability in selecting serums for establishment of cell lines from primary explants(12). The ability of established cell lines to multiply subsequently in most unselected serums(21) is most probably due to the selection of cell population capable of multiplying in higher concentration of toxic factor as demonstrated by Fredoroff(22).

The current status of this study is not inconsistent with the suggestion that both growth promoting and cytotoxic effects noted are the result of the relationship between 2 or more serum protein factors. Neither of these activities has been successfully concentrated in a subfraction of the active parent fraction, yet cytotoxicity, at least, can be regained by recombination of the subfractions.

It is also noteworthy that the growth promoting fractions of both human and equine se-

rums contain the major portion (50% or more) of the niacin of the parent serum which had been extensively dialyzed.

Summary. The essential growth factors associated with serum proteins are present in cold ethanol fraction IV-4 for most human serum pools and in fraction II + III for 2 individual equine serums. Cytotoxic activity has been found in fraction II + III from most human serum pools. Further subfractionation of IV-4 and II + III by the established cold ethanol methods fails to yield active subfractions. The significance of this finding is discussed.

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Received July 23, 1959. P.S.E.B.M., 1959, v102.

Is Multiple Sclerosis Caused by *Spirochaeta myelophthora* (Steiner)?* (25197)

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Ichelson(1) reported regular cultivation of a spirochete resembling *Spirochaeta myelophthora,* previously described by Steiner(2). She claims to have isolated from spinal fluid of 78% of 76 persons with clinical diagnosis of multiple sclerosis, a large spiral organism morphologically resembling members of the genus, Borrelia. In contrast she was unable to obtain a single positive culture from spinal fluid of 28 persons with diagnoses of other illnesses. We believed that several aspects of this report demanded clarification and/or confirmation. First, the report that one can cultivate "Borrelia-like" organisms from 78% of the spinal fluid of persons with multiple sclerosis regardless of stage of disease should be confirmed. Secondly, no exact data were included in the original report as to time following inoculation when each culture first be-

* Supported by grants from Multiple Sclerosis Fdn. of Am., Lewis J. Pollock, Responsible Investigator.

came positive. A third point open to discussion is the method of inoculation. In the illustrations accompanying the article by Ichelson are seen organisms which resemble some of the large Borrelia species resident in mouths of normal individuals. According to the author, inoculation of spinal fluid was made by blowing it into the medium with a Wright pipette. Since spirochetes are able to pass bacteriologic filters at times, could cultures have been established by passage of spirochetes through the pipette plugs by the force of blowing? This report presents results of our attempt to confirm Ichelson.

Materials and methods. The medium employed(1) contains Brewer's thioglycollate, asparagin, L-cystine, peptone, Wassermann-negative human serum, and rabbit serum, in a water diluent. This preparation was sterilized by filtration through a Selas 03 filter and was dispensed into tubes. Into the bottom of a tube of this medium there was delivered a few drops of the centrifugate from 10 ml of patient's spinal fluid. The inoculated tube then was overlaid with a paraffin oil-vaseline mixture. The tube was incubated at 30°C and examined once a week until growth took place. Two batches of medium were prepared adhering as closely as possible to Ichelson's(1) published methods. It was found impossible to prepare the medium as described by this worker, which was also the experience of others(3,4). Presence of agar in the thioglycollate broth caused a rapid plugging of pores of the Selas 03 filter. Consequently the Wassermann-negative human serum and aseptically-removed normal rabbit serum were filtered separately through such a sterile filter. The remainder of the ingredients were then prepared according to her directions, passed through sterile warmed M and UF grade sintered glass filters and then added aseptically to the serum mixture. Finally, all tubes of media were incubated for sterility for 3 to 5 days in the 37°C incubator and remained at room temperature for a week before inoculating them. Neurologists associated with Dept. of Neurology and Psychiatry, Northwestern University Medical School, obtained specimens of spinal fluid from patients who were either ambulatory or hospitalized at the affiliated hospitals of Northwestern University Medical School. Specimens were drawn aseptically into sterile, screw-capped tubes. Each specimen was kept at body temperature until delivered to the laboratory, the time between withdrawal of spinal fluid and its arrival at laboratory not exceeding 2 hours. Each specimen was transferred at once to a chemically clean, sterile, centrifuge tube and centrifuged at 1500 rpm for 2 hours (following Ichelson's procedure). One drop from the original sample tubes was examined immediately under a darkfield microscope for any animate or inanimate particles that might have some bearing on the problem. Such examinations were made with great care, by scanning many fields of each preparation. After 2-hour centrifugation, most of the supernatant fluid was removed, leaving only enough to allow for mixing with sufficient sediment to inoculate tubes and to make 3 additional slide preparations. One of these latter was examined by darkfield microscope, the second and third allowed to dry, stained by Gram and Giemsa methods respectively, and then examined. Inoculations were made into 3 tubes of broth-serum culture medium of Ichelson (2 tubes of one batch and 1 tube of a second batch of medium), and into 2 tubes of trypticase soy broth. The first serum-broth tube was inoculated by blowing the spinal fluid with the mouth from the pipette into base of the tube. The second was inoculated in the same manner, except that a bulb-manipulated pipette was used. A fresh bulb-manipulated pipette was substituted to inoculate the third tube. The trypticase soy tubes were inoculated, also, with a bulb-manipulated pipette. All serum-broth cultures were incubated at 30°C. One trypticase soy culture was left at room temperature, the other incubated at 37°C. These latter were intended to serve as sterility controls of cultures. Positive control tubes of serum-broth media were inoculated from stock culture of *Leptospira icterohemorrhagiae* (obtained from Dr. Evelyn B. Tilden, Brookfield Zoological Gardens, Brookfield, Ill.) to be sure that the medium would support growth of a known strain of spirochete. Since Ichelson did not state the total time over which she incubated her cultures, our cultures were kept for at least 6 months, examined at weekly intervals

for appearance of growth; grossly by transmitted light, and microscopically by darkfield method.

Results. Forty-two patients have been examined, 28 of whom are afflicted with multiple sclerosis, the remaining 14 with other conditions. It seems pertinent that some evidence be presented in support of the diagnosis of multiple sclerosis in these 28 patients. All were examined by an experienced Board certified neurologist(5). Of 28 patients listed as suffering from multiple sclerosis, 3 had a presenting syndrome of retrobulbar neuritis. Twenty-three were considered to have a progressive type of illness. Of 28 patients, 50% or more exhibited the following signs and symptoms: pyrimidal tract involvement, temporal pallor, nystagmus, bladder disturbance, ataxia, vibratory sense disturbance, diplopia, and position sense disturbance. Eleven of the group had speech difficulties. Twenty-four of these persons were between the ages 20-40 years; only 3 were in the 41-50 age group.

In 2 instances only did growth occur after inoculation of media previously described. This was probably contamination, since all inoculated tubes of the first specimen showed presence of Gram-negative bacilli; while the second revealed Gram-positive bacilli, along with minute amounts of broken cork pieces from the specimen tube cap lining. No spirochetes of any type were seen in any tube inoculated, except in the spirochete-positive control tubes.

Comparisons of examinations by darkfield, of material taken immediately from the specimen as soon as it reached the laboratory, and from material after it had been centrifuged for 2 hours, showed some variation. In some instances there were variable amounts of erythrocytes. The main difference in the centrifuged specimens (2 hours) consisted of the finding of leucocytes, which also differed in number from one, found after examining the whole specimen, to many/high power field.

The interesting thing about these white cells was their reaction to unfavorable environment, evidenced by their sending out various types of filipodia and/or pseudopodia from cell periphery. Sometimes the protoplasm would extend outward in very long strands, with a length of 10 to 15 times the diameter of the cell. These would wave about actively, eventually becoming pinched off in a manner such as to give the strand the appearance of an irregular string of beads. Some would break off from the parent cell and float about, disintegrating into smaller pieces, especially after pseudopodia became detached from the cell. Most of the movement was due to molecular motion of the preparation fluid. These bits of protoplasm appeared as brilliant white vibrating pieces which could very easily have been mistaken for a spirochete had not their origin been observed.

Erythrocytes usually appeared with many short extensions, about one-third the cell diameter, which gave them the appearance of being ciliated. These tiny filipodia were very mobile while attached to the cell.

The Gram stained films revealed nothing that was not already seen by darkfield examination; the Giemsa stained preparations showed leucocytes to be mostly lymphocytes, with only an occasional polymorphonuclear leucocyte. Eighty-six %(24) of the spinal fluids from cases of multiple sclerosis contained leucocytes which showed changes as described above. Of 14 spinal fluids from patients with diseases other than multiple sclerosis, only 5, or 25%, revealed such cells or their components. Patients in this group had been diagnosed as suffering from various disturbances such as cerebral vascular accident, Friedreich's ataxia, post-infectious polyradiculo-neuritis, psychomatic epilepsy, myasthenia gravis, hypertrophic osteoarthritis, and CNS lues. That our results might differ from those of Ichelson(1) so markedly by chance alone is less than one in a thousand when measured by the X^2 Method.

Summary. Using technics very similar to those proposed by Ichelson, a study was made of 28 persons with well documented diagnosis of multiple sclerosis, and 14 persons with other conditions which involved the central nervous system. We attempted to isolate a spirochete similar to the one which Ichelson claims to have found. We were unable to confirm her work. The possibility that this result could have occurred by chance alone is statistically invalid.

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Received June 1, 1959. P.S.E.B.M., 1959, v102.

Electrophoretic and Immunologic Studies of Rivanol-Fractionated Serum Proteins.*† (25198)

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Rivanol (6,9 diamino-2 ethoxyacridine lactate) has been employed by Hořejsí and Smetana(1,2) for precipitation of various protein fractions from serum or plasma at room temperature and in slightly alkaline solution. In contrast to most other procedures, *e.g.*, alcohol, salt fractionation, etc., addition of rivanol to serum does not result in precipitation of gamma, and to a lesser extent, beta globulins. This unique property of rivanol has been utilized in simple methods for preparation of highly purified gamma globulin(2), for separation of beta-1-metal combining globulin(3) and preparation of ceruloplasmin from Cohn's fractions III and IV(4). The apparent contradiction of the original concept of rivanol as specific reagent for separation of gamma globulin from all other serum protein fractions (2) and its use by others in preparation of beta globulin components led to the present study of rivanol-serum protein interactions. Our studies demonstrate that the supernatant solution, following rivanol precipitation of more rapidly migrating electrophoretic serum protein fractions, contains some beta globulin in addition to gamma globulin, as shown by both paper and moving boundary electrophoresis. In addition, this rivanol-containing supernate may be injected intravenously into rabbits resulting in antihuman-globulin sera of relatively high titer.

Materials and methods. Rivanol. Preparation of rivanol in these studies was obtained from Special Chemical Dept. of Winthrop Labs. under trade name "Ethodin." (Lot #N325FA). A 0.4% solution of rivanol in distilled water was prepared. *Rivanol-serum preparations.* To one volume of normal, human serum were added 1,2,3,5 and 9 volumes of 0.4% rivanol solution. The mixtures were left overnight at 4° and the resulting supernates were separated from precipitates for each mixture by centrifugation at 2250 g (International Type II) for 10 minutes. Supernates were then analyzed for protein composition by paper electrophoresis as described below. Corresponding precipitates were dissolved in 2 ml of citrate buffer (pH 5.0, 0.2 M). Except for small amount of insoluble material, the remainder of precipitate was readily soluble in this buffer. The redissolved precipitates were then subjected to paper electrophoresis in the same manner as their corresponding supernates. *Paper electrophoresis procedure.* The free-hanging horizontal strip technic of Dettker and Anduren(5) was used for paper electrophoresis determinations. The technic was essentially the same as described by Block, *et al.*(6), using Whatman No. 1 paper, barbital buffer (pH 8.6, 0.05 ionic strength) 220V for 6 hours and staining with Amido-Black 10B. Multiple aliquots of 10 lambda in inverse proportion to protein content were applied to paper strips (Fig. 1).

* Aided by Grant from U.S.P.H.S.

† The authors wish to acknowledge the aid of Shirley Gerstenfeld in the paper electrophoretic patterns, of Francis Vecsler, in performing moving boundary electrophoresis experiments, and Lillian Salowitz in editing and typing of manuscript.

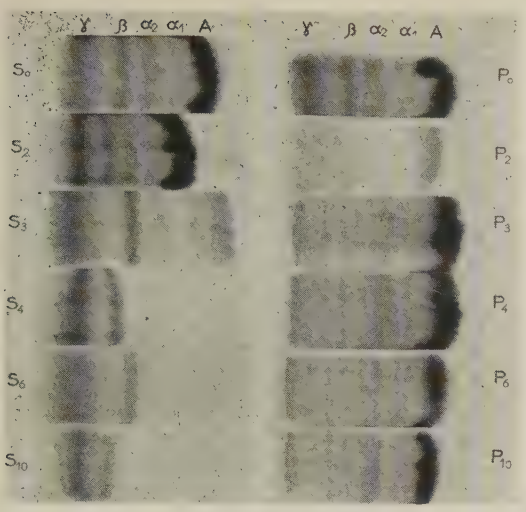

FIG. 1. Paper electrophoretic patterns obtained with barbital buffer (pH 8.6, .05 ionic strength), of the supernates and precipitates (dissolved in citrate buffer pH 5.0) of various rivanol-serum ratios. S_0, P_0 represents aliquots of original serum sample. S_2, P_2 S_3, P_3, etc. represent supernates and precipitates obtained by diluting serum 1:2, 1:3, etc. with .4% rivanol. Different aliquots of each sample were applied to the paper in inverse ratio to their protein content.

Samples of untreated serum were run simultaneously with both supernates and precipitates. Study of immunologic properties of rivanol-globulin complex employed 4 rabbits. Two of these were injected 4 times/week for 4 weeks, and 2 received subcutaneous doses once each week for same period. The dose was kept constant at 5 ml of supernatant solution derived from 1 vol. of serum mixed with 5 volumes of rivanol (1:6). In neither group were any untoward reactions observed, either local or generalized. Animals were bled weekly *via* ear vein, sacrificed at end of 4th week and autopsies performed. Serum obtained from weekly bleeding was titered against a 0.1% solution of purified human gamma globulin as the antigen.

Results. The electrophoretic runs (Fig. 1) demonstrate that as volume of 0.4% rivanol solution is increased in relation to a fixed volume of serum, the faster migrating electrophoretic fractions (albumin and alpha-globulins) are progressively removed from the supernate and appear in corresponding precipitates. In a 1:4 mixture the supernate contains only gamma and beta globulins. Further addition of 0.4% rivanol solution does not result in separation of any additional fractions as indicated in S_6, P_6, and S_{10}, P_{10} patterns. When a 0.2% rivanol solution was used instead of 0.4%, a mixture of 1:6 or higher was required to obtain the supernate pattern corresponding to S_4 above. An analysis of the supernate equivalent to S_6 (Fig. 1) with the Tiselius moving-boundary electrophoresis (barbital buffer, pH 8.6, 0.1 ionic strength) showed presence of components in the following relative concentration (as % of the total protein): – gamma globulin (79%): beta globulins (13%) and alpha globulins (8%).

Autopsy of rabbits injected both intravenously and subcutaneously with the rivanol-globulin supernate revealed no gross abnormalities of any of the viscera. Microscopic examination of lung, liver, spleen, lymph node, kidney and adrenal showed no unusual features as compared to those seen in the usual process of immunization.

A progressive rise in precipitin titer to as high as 1:600 at end of 4 weeks, was obtained in sera of intravenously treated animals while subcutaneously injected animals showed a titer of 1:32. There was no cross precipitation with human albumin.

Discussion. Our study confirms the work of previous investigators(1,2,3,4) that addition of rivanol to serum or plasma at room temperature results in selective precipitation of protein fractions of greater mobility and retention in solution of those of lesser mobility. From our studies as well as previous studies of Hořejsí and Smetana(1,2) and of Boettcher, *et al.*(3), it appears that the major protein fractions remaining in the supernate at optimal rivanol (0.4%)-serum mixtures (>4:1) are gamma globulin and beta-1-metal combining globulin. The probable reason for lack of reactivity of many proteins, *e.g.*, gamma globulin, with organic cations and anions has been explained by Klotz(7,8) as due to internal binding between hydroxyl amino acids and carboxylic and cationic amino acid side chains supplemented by Van der Waals forces. In our opinion this does not rule out the possibility of soluble complex formation between these 2 protein fractions

and rivanol; this is being investigated.

The problem of possible denaturation and loss of biological activity of gamma globulin prepared by rivanol treatment has been extensively investigated by Hořejsí and Smetana (2) with respect to electrophoretic mobility, polarographic and potentiometric determination of SH groups, dye binding, viscosity and tryptic digestion. Of particular interest was their demonstration that rivanol prepared gamma globulin contained antibodies against polioencephalitis, several strains of influenza virus and infectious hepatitis of the same degree of protective capacity as did ethanolic preparations of gamma globulin. These results, which demonstrate unimpaired antibody activity of rivanol prepared gamma globulin, are supplemented by our finding of potent antigenic activity of such gamma globulin preparations. We have also shown that it is not necessary to remove rivanol prior to parenteral injection and that it is without deleterious effect on rabbits. Use of supernate without further purification, *e.g.*, charcoal absorption to remove rivanol, prevents loss of considerable proportion of original serum gamma globulin content.

While it is well known that most antibodies are gamma globulins, a number of antibodies have also been shown to have electrophoretic mobilities corresponding to beta-2-globulins (9). Therefore, the admixture of beta globulins with gamma globulins resulting from rivanol preparations may be advantageous, as for example in the immunohistochemical study of hypersensitivity states. It appears from the work of Boettcher, *et al.*(3), as well as our preliminary studies that the beta globulin component obtained with higher rivanol-serum ratios is the beta-1-metal combining globulin (transferrin or siderophilin). This component has recently been reported to possess antiviral activity(10).

These studies are being pursued along 2 parallel lines. Firstly, the antigenic properties and potency of various rivanol-plasma protein fractions are being investigated. Secondly, more detailed physico-chemical studies of these fractions are being carried out.

Summary. 1. Variation of ratio of 0.4% rivanol (6,9, diamino-2 ethoxyacridine lactate) solution to that of serum, provides a simple and rapid method for fractionation of serum proteins at room or refrigerator (4°) temperature. 2. Paper electrophoresis (barbital buffer, pH 8.6, 0.05 ionic strength) was used to determine protein fractions remaining in the supernate and those found in corresponding precipitates (soluble in pH 5.0 citrate buffer) at various serum-rivanol ratios. 3. The supernate of serum-rivanol mixtures of 1:4 or higher consisted almost entirely of gamma globulin (79%) and of the beta-1-metal combining globulin (13%) as determined by moving boundary electrophoresis. 4. Immunization of rabbits by direct intravenous injection of rivanol-globulin supernate caused no deleterious effects in animals and resulted in high titer antisera (1:600). 5. The possible advantages of using rivanol dilution as a simple method for preparation of concentrated plasma protein fractions containing substances with specific biological activity, *e.g.*, ceruloplasmin, gamma globulin, etc. is discussed.

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Received June 25, 1959. P.S.E.B.M., 1959, v102.

Experimental Variation in Mouse Virulence of Echo 9 Virus. (25199)

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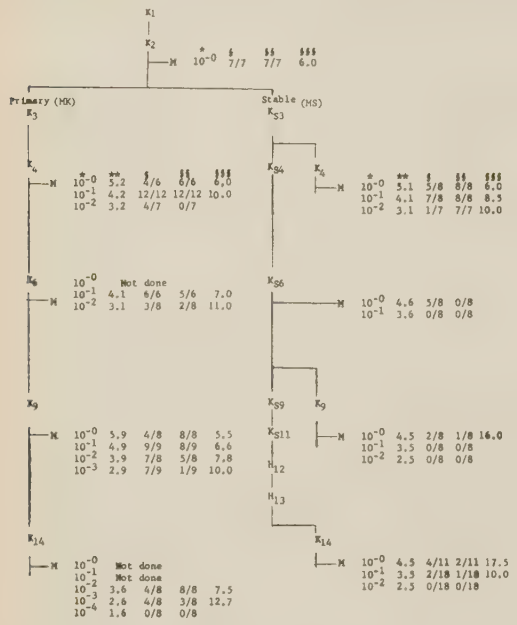
As reported previously mouse virulence of type III poliovirus (mouse-adapted) grown in tissue culture (TC) depended on the host cell and its maintenance medium. When grown in monolayer TC in Medium 199, monkey (Rhesus) kidney (MK) cells enhanced mouse virulence while HeLa cells tended to inhibit it(1). Many other serial cell lines including monkey stable (MS) kidney cells, possessed the same inhibitory property(2). The present study was conducted to see whether mouse virulence of Echo 9 virus would be influenced by the host cell in a similar way.

Materials and methods were similar to those described previously(1) except for the following modifications. Echo 9 virus, Bourn strain (3) was serially passed in monkey (Rhesus) kidney tissue culture (MKTC), plaque purified once, and again transferred to MKTC by Drs. J. L. Melnick and Y. Kanda. This virus (Bourn LR $451K_8$ P_1 K_1) was used and was designated as K_1. The K_1 virus was again transferred to MKTC (K_2, Chart 1), and from which 2 lines of serial MKTC passages were carried, (a) in primary cells and (b) in MS cells(5), all fed with Eagle's medium with 2% calf serum. For initiating the serial passage, 0.2 ml of the undiluted TC fluid was inoculated to each of 4 TC tubes, each containing 1.5 ml medium. At the end of the experiment, as MS cells were not available, HeLa cells were used instead for the last 2 passages. The virus from the primary cells was harvested usually on the 4th or 5th day when most of the cells were destroyed while that from MS cells on the 8th or 9th day as cytopathic effect was greatly delayed in the MS cells. At various passage levels the virus was titrated in TC as well as assayed in mice. Titration in TC was made by inoculating 0.2 ml of each of serial 10-fold dilutions into primary MKTC in Medium 199 with 2% calf serum, using 6 tubes per dilution. Assay of virulence was made in 1-day-old Swiss mice by intramuscular inoculation of 0.02 ml of different dilutions, using 1 or 2 litters of mice per dilution each litter consisting from 5 to 9 mice. As titer of virus from the MS cells was relatively low, the virus was often transferred to primary cells in Eagle's medium with 2% calf serum for 1 passage in an attempt to raise its titer before virulence assay, (K_9, K_{14}, Chart 1).

Results. These are summarized in Chart 1. Before the experimental passages the virus was found virulent as all 7 mice inoculated with undiluted (not titrated) TC fluid died in 6 days (average). After one passage the viruses (K_4) grown in either primary or MS cells were all virulent for the suckling mice. After further passages, however, the virus grown in primary cells remained consistently virulent as tested at passage level K_6, K_9, and K_{14} while the virus grown in MS cells and tested at comparable passage levels lost most of its virulence as indicated by paralysis rate, death rate and survival time of those mice which eventually died. Although titer of virus from MS cells was relatively lower, the difference in virulence between the 2 lines was not due to the quantity of virus in the inoculum. For instance, at passage level K_{14}, a dose of $10^{3.6}$ $TCID_{50}$ virus from primary cells killed all 8 inoculated mice in 7.5 days (average) while a bigger dose ($10^{4.5}$ $TCID_{50}$) of virus from MS cells killed only 2 out of 11 mice in 17.5 days (average). (In the later group, 4 out of 11 mice became paralyzed but 2 of them recovered.)

Discussion. These experiments demonstrated clearly that the mouse virulence of Echo 9 virus could be definitely influenced by the host cell in which the virus was grown. This phenomenon was somewhat similar to but not identical with that observed in a study of type III poliovirus, the mouse-adapted Leon strain. In that study(2), the mouse virulence of Leon virus was nullified after 2 passages in MS cells in lactalbumin or bovine plasma hydrolysate medium(4). In the present study virulence of Echo 9 virus was reduced after several passages in the MS cells

CHART 1. Assay of virulence in mice of Echo 9 virus after serial passages in primary or stable monkey kidney tissue culture.



Key for Chart 1

K = Primary monkey kidney tissue culture passage. K_S = Stable monkey kidney tissue culture passage. K_1; K_S; K_{S3}; etc. = First passage in primary monkey kidney tissue culture; third passage in primary monkey kidney tissue culture; third passage in stable monkey kidney tissue culture. M = Mice inoculated intramuscularly. * = Dilution of tissue culture fluid, $\log_{10}$. ** = Log No. of $TCID_{50}$ inoculated into one mouse. § = No. of mice paralyzed or sick over No. inoculated. §§ = No. of mice died over No. inoculated. §§§ = Average survival time (days) of those mice which eventually died.

in Eagle's medium, while virulence was not reduced by passage in MS cells in bovine plasma hydrolysate medium. Kanda and Melnick found that 12 serial passages in MS cells did not affect the monkey neurovirulence of type I poliovirus, (Mahoney)(5). In the present study intracerebral inoculation of 1 ml of undiluted virus (passage level K_{12} and K_{S12}) into each of 4 rhesus monkeys—2 monkeys for virus of each cell line—produced no symptoms or rise of temperature during a period of 21 days nor histopathological lesions in their central nervous system afterwards. Mouse virulence seemed to be independent of monkey virulence.

The present study was aimed at demonstrating that the dependence of viral virulence on the host cell was not confined to type III poliovirus. No attempt was made to purify the Echo 9 virus by the plaque method as was done for the type III poliovirus(2). How the MS cells selected the avirulent or less virulent virus particles was not determined.

The MS cells were found to be harboring pleuropneumonia-like organisms (PPLO) which might, it was thought, influence the virulence of virus. However, in separate studies, IC inoculation of a pure culture of PPLO (10^4 organisms per mouse) did not cause symptoms or death in suckling mice; nor did it influence the activity of a tumor-inducing virus (unpublished). The virus grown in either primary or stable cells was neutralized in TC by anti-Echo 9 rabbit serum. Eggers and Sabin(6) have extended the work of McLean and Melnick(3) and other workers and have reported that the naturally occurring Echo 9 viruses varied in mouse pathogenicity. The virulence problem is complicated and probably a large number of determining factors are involved.

Summary. Serial passages of Echo 9 virus were carried out in 2 sets of monolayer tissue culture in Eagle's medium: (a) in primary monkey kidney (MK) cells (b) in monkey stable, (MS) kidney cells. After several passages, the virus lost most of its virulence for mice in MS cells but not in MK cells.

The author wishes to thank Drs. J. L. Melnick and Y. Kanda for supplying Echo 9 virus and stable monkey kidney cells; to Dr. K. Habel and Miss R. J. Silverberg for supplying anti-Echo 9 serum; to Drs. Van Hoosier and R. L. Kirschstein for monkey experiment and histopathological studies; and also to J. L. Rogers and E. W. Harvey for technical assistance.

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Received July 13, 1959. P.S.E.B.M., 1959, v102.

Formation of Acid Glycoproteins in Serum.* (25200)

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The perchloric acid-soluble protein fraction in serum, often designated as the seromucoid or the mucoprotein fraction(1,2), is a protein fraction of high carbohydrate content. In humans it comprises less than 1.5% of the total proteins, but about 10% of the total protein-bound carbohydrate. The major component of this fraction is identical with the acid α_1-glycoprotein isolated and characterized by Weimer, Mehl and Winzler(3) and by Schmid(4). Characteristic of this glycoprotein is the high sialic acid content(5), which is responsible for the low isoelectric point. The small-molecular α_2-glycoproteins of Schmid(6) are probably also contained in this fraction. The acid glycoprotein fraction is of clinical interest, since it has been shown that its concentration increases in serum in a variety of neoplastic and infectious conditions (1). Therefore it has often been assumed that the increase in this fraction is in some way associated either with tissue destruction or with cell proliferation. The possibility that the liver is involved in production of acid glycoproteins is suggested by the observations by Greenspan *et al.*(7,8) that content of this fraction in serum decreases in uncomplicated parenchymatous liver diseases. In rheumatoid arthritis there is a marked increase in acid glycoproteins, serum level being significantly correlated to activity of the disease(9). We found(10) remarkably high concentrations of acid glycoproteins also in synovial fluid of patients with active rheumatoid arthritis. The values were almost as high as in serum in spite of the much lower total protein concentration in the synovial fluid. These data suggest the possibility that in arthritis there might also be a localized production of acid glycoproteins in the inflamed synovial tissue. It seemed of interest, therefore, to study the rôle of the liver in formation of acid glycoproteins both under normal conditions and in experimental arthritis. The effect of carbon tetrachloride-induced damage to the liver was therefore studied. Since the extensive liver injury produced by this agent also could be expected to affect the reticulo-endothelial system (RES) in the liver, control experiments with the RES blocking agent Thorotrast were included. To estimate extent of liver damage, serum GPT (glutamic puruvic transaminase) content was determined. The effect on the function of the RES was followed by determinations of prothrombin and proconvertin. Recent results by Slätis(11) strongly indicate that these coagulation factors are synthesized in the RES.

Materials and methods. All experiments were carried out on white male rabbits weighing 2.5 - 3.5 kg. Blood was collected from the marginal vein of the ear by piercing the vein with a lancet after the ear had been rubbed with xylene for about a minute. Care was taken not to rub the site of incision in order to avoid hemolysis. Usually about 6-8 ml blood was collected. In view of the findings of Werner(12) that serum hexosamine in rabbits increases after repeated bleedings control experiments were done in order to find out whether the withdrawal of blood might influence the acid glycoprotein content of serum. Four rabbits were bled 30-40 ml daily for 2 days and determinations of total protein and acid glycoproteins were made 24 hours after the second bleeding. No significant change in the acid glycoproteins was found (average change + 2%) whereas total protein content decreased on an average 15%, ($P<0.01$). Carbon tetrachloride was administered through a stomach tube in doses of 2 ml per kg body weight. Thorotrast (Testagar & Co., Inc., Detroit, Mich.) was injected into the marginal vein of the ear using 4 ml per kg body weight. Arthritis was produced by intra-articular injection (left knee joint) of 0.8-1.2 ml of a suspension containing about 10^9 living bacteria per ml. Experiments were performed with a strain of *staphylococcus*

* This investigation was supported by a research grant from Gustaf V 80-years Fn.

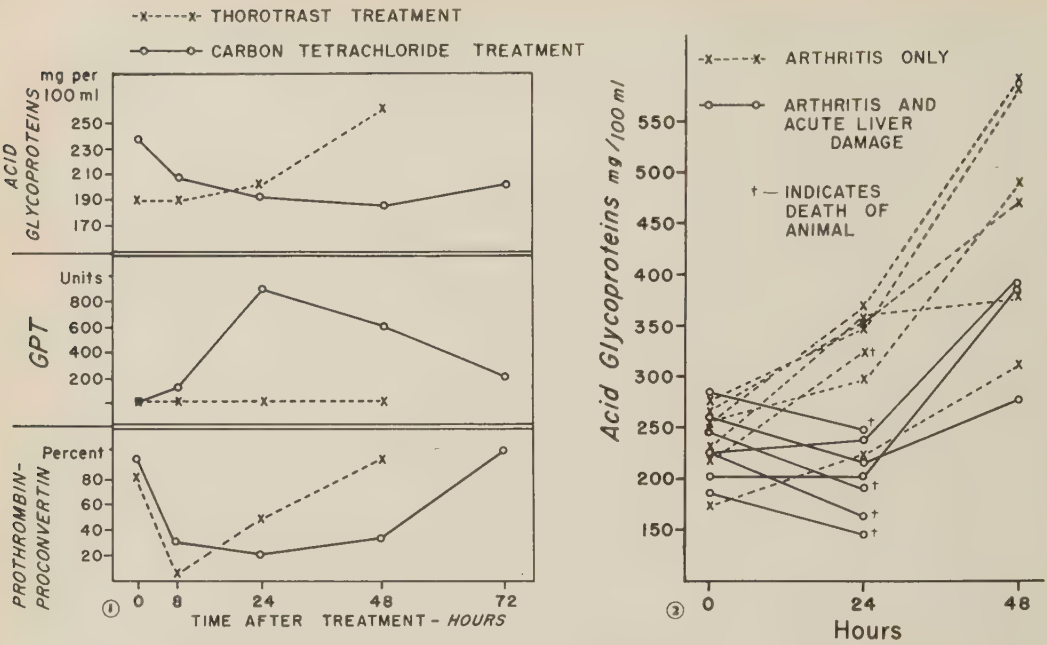

FIG. 1. Changes in acid glycoproteins, GPT and prothrombin-proconvertin following RES blockade (Thorotrast) and induced liver damage (carbon tetrachloride). Three rabbits in each group; points represent average values. Thorotrast (4 ml/body weight) was administered intravenously and carbon tetrachloride (2 ml/kg body weight) through stomach tube.

FIG. 2. Effect of acute liver damage on acid glycoprotein response in experimental arthritis. Arthritis produced by intra-articular injection of suspensions of living bacteria. At the same time carbon tetrachloride was given to 7 animals. Average percentage change in acid glycoprotein concentration after 24 hr: Arthritis only ($n = 7$) $+ 34.4\%$; $t = 7.56$; $P < .001$. Arthritis + CCl_4 ($n = 7$) $- 13.1\%$; $t = 3.17$; $P < .05$. Difference between groups: 47.5 ± 6.1 ($P < .001$).

aureus and with a strain of pneumococcus (Type IV). With both bacterial suspensions signs of arthritis, limping and impaired mobility, were present one day after injection. Acid glycoproteins were determined according to a modification(13) of the method of Winzler *et al.*(14) and are expressed in mg/100 ml serum. For 20 normal rabbits an average value of 245 ± 10 mg/100 ml serum (standard deviation : 43) was obtained. Prothrombin and proconvertin (P & P) were determined by a micro modification of Owren's method(15) and expressed as % of average value found for normal rabbits. Glutamic puruvic transaminase (GPT) was determined according to Frankel and Reitman(16) and expressed in Karmen units.

Results. Fig. 1 shows a comparison between the effect of RES blockade and acute liver damage in 2 groups each comprising 3 animals. The response was similar for all animals within each group. The marked rapid decrease in the P & P values following administration of Thorotrast is similar to the effect obtained by Slätis on rats(11). The coagulation factors almost disappeared within 8 hours and later rose almost as rapidly to values slightly above the initial ones. The absence of increase in transaminase values suggests that Thorotrast had no significant toxic effect on the liver. There was no significant change in acid glycoprotein concentration of the serum during the first 24 hours but after 2 days there was a moderate increase. The concentration returned to normal 2 weeks after Thorotrast injection.

With carbon tetrachloride the P & P values also decreased, although not to the same extent as with Thorotrast. The change paralleled the increase in GPT rather closely. The acid glycoproteins also decreased slowly reaching a minimum after 48 hours. The decrease at this time was found to be statistically significant (average decrease for 6 ani-

mals = 17.3%; t = 5.20; $P<0.01$).

Fig. 2 shows the marked increase, 90% after 48 hours, in acid glycoproteins following intra-articular injection of suspensions of pneumococcus (Type IV) or *Staphylococcus aureus*. The results were essentially similar with both types of bacteria. When carbon tetrachloride was administered simultaneously with injection of bacteria, acid glycoprotein concentration decreased after 24 hours in 6 of the 7 cases. The difference between acid glycoprotein concentration of the serum in the 2 groups is highly significant.

All animals survived the arthritis with the exception of one, which died 2 days after injection. Bacterial injection combined with carbon tetrachloride poisoning caused the death of 4 of the 7 animals on the second day. Histological examination of the liver showed very extensive necrotic changes. It is also evident from Fig. 2 that in the surviving animals acid glycoprotein concentration increased rapidly.

Discussion. Administration of carbon tetrachloride to rabbits produced acute liver damage manifested by rapidly increasing serum transaminase values. Since the RES of the liver may be affected by this treatment the function of this system may also be impaired. Evidence for this was obtained by a decrease in the prothrombin-proconvertin test values following carbon tetrachloride. It was nearly as marked as that obtained by RES blockade with Thorotrast. On the other hand Thorotrast produced no increase in GPT.

Since carbon tetrachloride, in contrast to Thorotrast, produced a significant decrease in the acid glycoproteins, it seems likely that this effect should be attributed to liver cell injury and not to the effect on the RES.

Experimental arthritis produced a rapid increase in the perchloric acid-soluble protein fraction of a degree similar to that observed in arthritis in man. The results (Fig. 2) clearly indicate that the initial rise in this protein fraction following infection should be ascribed to increased production in the liver. However, it was found that, in those animals which survived the massive dose of carbon tetrachloride for more than one day, acid glycoproteins started to rise again on the second day. The most likely explanation for this observation is that a fast restoration of some liver functions occurs. That some localized production of acid glycoproteins may also have occurred can, of course, not be excluded by the experiments reported here.

The results support the view gained from previous clinical studies that the liver is the site of formation of the acid glycoproteins in serum.

The increase in acid glycoprotein production as a response to infection is remarkably rapid and serum concentration may rise to more than twice the initial value in 48 hours. The recent results of Boström *et al.*(17) also indicate that the turnover of the acid glycoproteins is considerably higher than that of other plasma proteins.

The physiological rôle of the acid glycoproteins is still unknown. Werner(12) observed an increase in serum hexosamine, α- and β-globulins after repeated large bleedings in rabbits and suggested that the synthesis of carbohydrate-rich proteins is associated with increased plasma protein synthesis. In similar bleeding experiments we found however no significant change in the acid glycoprotein concentration in spite of a significant decrease in total protein concentration.

In clinical studies it has on the other hand been demonstrated that changes in the acid glycoproteins are regularly associated with other changes in the serum protein pattern (18). Notable is the negative correlation between acid glycoprotein and albumin content which is especially marked in rheumatoid arthritis. These observations may indicate that the acid glycoproteins of the serum are in some way associated with albumin synthesis.

Summary. Acid glycoproteins of serum have been studied in experimental arthritis, after carbon tetrachloride poisoning and after RES blockade with Thorotrast, in rabbits. The results indicate that liver cells are the site of formation of the acid glycoproteins.

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Received July 21, 1959. P.S.E.B.M., 1959, v102.

Temperature Control During Homogenization.* (25201)

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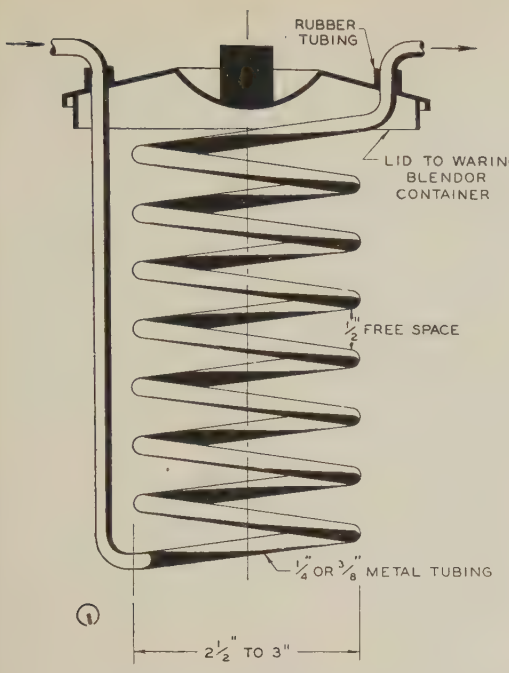
Length of time for which biological materials can be subjected to high speed homogenization is frequently limited by sharp rises in temperature which accompany such treatment. These temperature increases are particularly severe with fibrous tissues, and have also been observed during disruption of microorganisms by agitation with fine glass beads in the Waring Blendor(1). Such temperatures not only destroy labile cellular constituents, but constitute a safety hazard when volatile, flammable solvents are used. Temperature control is particularly difficult in homogenizers whose drive unit is located beneath the container, since the container can not be packed in ice or cooled in a water bath. Procedures commonly used for controlling homogenate temperatures in instruments such as the Waring Blendor include pre-cooling the container, working in the cold room, alternately homogenizing and chilling the container, and dropping ice into the homogenate. These measures frequently are not only either slow or inconvenient, but are ineffective when prolonged homogenization is required. With apparatus modifications and procedures described below, it is possible to maintain low temperatures during extended homogenization. The necessary equipment modifications may be made on standard homogenizer containers; these alterations are simple, inexpensive, and require only materials and tools available to most laboratory shops. Although methods and results described below are for the Waring Blendor, it should be feasible to adapt them to other makes of homogenizers. Evidence for the effectiveness of apparatus and procedures is presented in this report.

Methods. Temperature control in glass Waring Blendor containers is readily obtained by use of an internal cooling coil of the type shown in Fig. 1. During construction, care must be taken (a) not to collapse the tubing when winding the coil and (b) to position the coil so that it clears the blades by a safe distance. When coil is in use, water, a few degrees cooler than the temperature desired in the homogenate, is circulated through it by a small centrifugal pump. *Temperature control in semi-micro containers.* While the internal cooling coils described above are suitable for large glass and metal homogenizer containers, they cannot be used in smaller semi-micro units. Temperature control in these containers, however, is easily obtained by installation of water jackets. Fig. 2 shows a metal semi-micro container for the Waring Blendor fitted with cooling jacket made from 3-inch diame-

* Supported in part by grant from Nat. Inst. of Allergy and Infect. Dis.



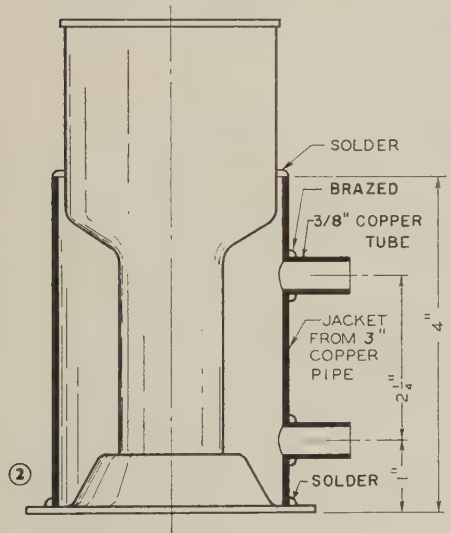


FIG. 1. Metal cooling coil for use in glass containers for Waring Blendor.

FIG. 2. Cooling jacket for metal semi-micro Waring Blendor container.

ter copper pipe. The shape of container renders it unnecessary to construct either a top or bottom for the jacket. Inlet and outlet tubes of 3/8-inch diameter are first installed in the copper pipe and fastened either by soldering or silver brazing. Because the container is itself fabricated with a brazed joint, it is not advisable to attempt to braze the jacket to the container, since the heat required might damage this joint. The jacket, with inlet and outlet tubes in place, is therefore joined to the container with ordinary soft solder and carefully controlled heat. Blade assembly and gaskets are removed prior to soldering. During homogenization, cooling is accomplished by circulation of cold water with a small centrifugal pump.

Results. With either the jacketed semi-micro container or the glass container with internal cooling coil, we have consistently kept homogenates at 2°C to 7°C by circulating water at 0°C. Both modified containers have proved their superiority over unaltered containers in maintaining low temperatures, even when the unaltered containers were pre-cooled to −10°C and operated in the cold room. Efficiency of the jacketed container is indicated by the data in Fig. 3. The internal cooling coils were similarly effective: in a representative experiment, the temperature of a homogenate of cotton leaves (75 g in 350 ml water) rose from 3.2°C to only 6.5°C during

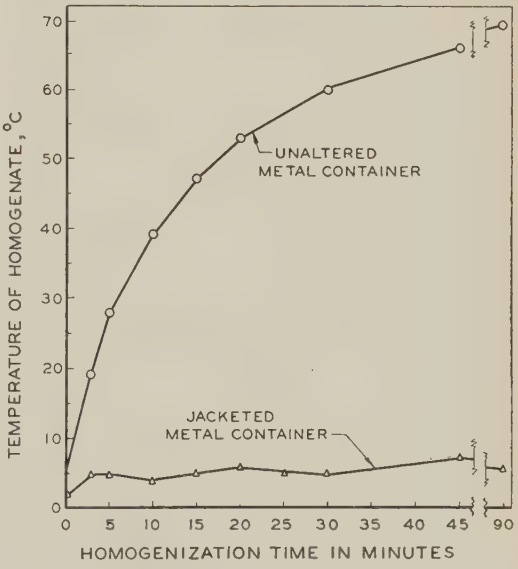


FIG. 3. Temperatures observed during disruption of yeast cells by agitation with small glass beads in jacketed and unaltered metal semi-micro containers for Waring Blendor. Cooling water at 0°C was circulated through the jacketed container. The unaltered container was pre-chilled in the freezer and kept in cold room at 11°C during agitation. Mixture consisted of 100 g of glass beads, 50 g yeast, and 100 ml water.

3 hours of homogenization, while the temperature of an identical sample in a container without cooling coil increased from 0.5°C to 90°C after only 1 hour.

Internal cooling coils did not interfere with effective homogenization, and proved to be more efficient in controlling temperatures than water jackets on the glass containers. Jackets for these containers constructed from large polyethylene bottles and cemented in place with epoxy resin were only moderately successful because of poor heat transfer through the thick glass walls. When the cooling coil is used during homogenization in the presence of abrasive materials (such as small glass beads) there is an etching effect on the lowermost helix, and this section should be protected by a sleeve of plastic tubing. Cooling coils of ⅜-inch tubing were found to be more satisfactory than those of smaller size because of the faster flow rate of cooling water which they permit. We have used both aluminum and copper tubing; stainless steel may be employed where conditions require it.

Summary. Effective cooling during prolonged homogenization at 15,000 r.p.m. in the Waring Blendor was obtained by simple, inexpensive modifications to standard Waring Blendor containers. Homogenates in glass containers were cooled by insertion of coils made of metal tubing through which ice water was pumped; metal semi-micro containers were modified by installation of water jackets for circulation of a coolant.

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Received July 7, 1959. P.S.E.B.M., 1959, v102.

Viral Sensitivity of Cell Cultures Maintained with Skim Milk Medium. (25202)

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Development of a serum-free medium containing skim milk, which effectively maintains a number of cell cultures(1), prompted this study. Previous data showed that heat sterilization of milk in preparation for this medium completely destroyed any antibodies that might be present(1). The viral sensitivity of cell cultures in presence of skim milk maintenance medium was compared to sensitivity of the same cell lines on an established medium.

Material and methods. Virus. Virus strains and host cell lines are listed in Table I. *Cell cultures* employed were prepared in the cell culture laboratory, Division of Biologics Standards, Nat. Inst. of Health by Mr. George Gardner. Primary monkey kidney cells(2) were propagated in medium 199(3) containing 2% calf serum* and antibiotics† and maintained in medium 199 containing 20% skim milk at 30°C until inoculated with virus. All other cell lines were held at 36°C. HeLa cells(4) were propagated and maintained in medium 199 containing 10% calf serum. Primary rabbit kidney cells(5) were propagated in lactalbumin hydrolysate medium(6) containing 10% calf serum and maintained in basal medium Eagle (BME) (7) containing 10% calf serum. HEP II cells(8) were propagated and maintained in BME containing 20% calf serum. *Viral titrations* were performed in duplicate in each cell line. Cell cultures of 1 titration were fed medium 199 containing 20% skim milk. Cell cultures of second titration were fed as follows: monkey and rabbit kidney cells—medium 199; HeLa cells—tryptose phosphate broth medium (TPB)(9) consisting of 70% medium 199, 25% tryptose phosphate broth and 5% chicken serum; HEP II cells—BME

* All serum used for cell culture medium was inactivated at 56° for 30 min.

† The following concentration of antibiotics was used: 100 units of penicillin/ml, 100 μg of streptomycin/ml, 100 μg of mycostatin/ml (not used in maintenance medium).

TABLE I. Viral Infectivity Titers (Log_{10}) in Presence of Skim Milk Medium and Established Maintenance Medium.

Virus	Cell line	Mean titers $TCID_{50}$/0.2 ml: Established medium	Skim milk medium
Polio I (Mahoney)	P. monk. kid.*	7.6 (199)	7.6
II (MEF)	*Idem*	7.4 "	7.4
III (Saukett)	"	6.9 "	6.9
Echo 1	"	7.3 "	7.7
10	"	7.3 "	7.3
19	"	7.2 "	7.5
Coxsackie A7	"	6.8 "	6.5
A9	"	7.5 "	7.8
B1	"	5.7 "	6.3
B2	"	5.7 "	5.9
B3	"	6.5 "	6.5
B4	"	5.7 "	6.2
B5	"	6.8 "	7.3
Vaccinia	"	5.4 "	5.4
Influenza A (Asian)	"	4.8 "	4.8
A′ (Denver)	"	3.6 "	3.3
Coxsackie A-11	HeLa	2.5 (TPB)	4.5
Adeno 3	"	7.8 "	6.8
4	"	6.9 "	5.7
7	"	7.3 "	6.3
Herpes simplex	P. rab. kid.†	7.0 (199, 5% c.s.‡)	7.0
Measles	H. Ep. II	2.8 (BME, 5% ch.s.§)	2.8

* Primary monkey kidney.
† Primary rabbit kidney.
‡ c.s. = calf serum.
§ ch.s. = chicken serum.

containing 5% chicken serum. All cell cultures were washed 3 times with balanced salt solution before addition of assay medium. Serial 10 fold dilutions of virus were made in balanced salt solution and 3 to 8 cell culture tubes/dilution were inoculated with 0.2 ml of virus. All titrations were held at 36°C for 7 days except adenovirus and measles which were refed at 7 days and held at 36°C for 14 days. If 50% or more of the cells had undergone cytopathogenic change, the cell culture was considered infected. The ID_{50} end point was calculated by the method of Reed and Muench(10).

Results. Titrations showed no significant decrease in virus titer with use of skim milk medium as compared with the established medium for 19 of 22 viruses tested. Adenovirus 3, 4 and 7 showed a 1 log_{10} lower titer in skim milk than in TPB medium. Coxsackie A-11 titers were 2 log_{10} higher in skim milk than in TPB medium. Table I shows these results.

To determine whether skim milk acts directly to repress adenovirus multiplication or lacks the cell-mediated enhancing factor of tryptose phosphate broth(9), a number of experiments were performed: 1. Different combinations of constituents of TPB medium and skim milk medium were used for maintenance of cell cultures during titrations. 2. Sufficient skim milk to provide a 20% concentration was added to adenovirus titrations incubated 24 hours at 36°C in medium 199. 3. A 1:10 dilution of adenovirus made in 100% skim milk and a 1:10 virus dilution made in TPB medium were incubated at 36°C for 2 hours. Ten-fold dilutions of these incubated samples were made in TPB medium and inoculated into cell cultures maintained in TPB medium.

The following comparisons may be made from results shown in Table II. 1. In confirmation of results obtained by Ginsberg, *et al.*(9), tryptose phosphate broth enhanced the virus titer (col. 1 & 2); and use of TPB medium consistently resulted in higher titer of adenovirus than the other mixtures. 2. Addition of tryptose phosphate broth or chicken serum to skim milk medium elevated the titer somewhat above that obtained with skim milk medium (col. 3, 4 and 5). However, these elevated titers did not equal those recorded with use of TPB medium, indicating that the lower titers with skim milk were not entirely due to lack of tryptose phosphate broth. 3. Skim milk medium containing both tryptose phosphate broth and 5% chicken serum resulted in no higher titers than skim milk with either of the components (col. 4, 5 and 6). This medium may also be regarded as TPB medium containing 20% skim milk in which the virus titer was decreased by addition of milk. In this case, the action may be due to presence of a viral inhibitor in the skim milk or failure of skim milk medium to maintain the cells in optimal condition for virus multiplication. 4. Virus incubated 24 hours in cell cultures with medium 199 before skim milk was added, did not attain the titer reached by virus in TPB medium (col. 1 and 7). 5. No significant difference in titer was noted with virus incubated in 100% skim milk and

TABLE II. Effect of TPB Medium, Skim Milk Medium and Various Combinations of Their Components on Adenovirus Titers (Log_{10}) per 0.2 ml.

		$TCID_{50}$ in presence of								
		1	2	3	4	5	6	7	8	9
Virus	Cell culture	TPB*	199, 5% ch.s.	SM†	SM, 5% ch.s.	SM, 25% tpb‡	SM, 5% ch.s., tpb	199 (sm§ added after 24 hr to make SM)	TPB (virus incub. 2 hr in SM before dilution)	TPB (virus incub. 2 hr in TPB before dilution)
Adeno 3	HeLa	7.8		6.3						
		8.2		7.2						
		7.5		7.0						
Adeno 4		7.0		5.8	6.5	6.3	6.3			
		7.3		6.5	"	6.8	6.8			
		6.8		5.6				5.9	7.0	7.0
	H.Ep. II	"	6.3	5.3	5.3	6.0	6.0	5.3	6.5	6.5
		"	6.0	5.5	6.0	6.3		6.0	7.0	6.8
Adeno 7	HeLa	7.3	5.8							
		"	6.5							
		"	"							

* TPB = Tryptose phosphate broth medium. † SM = Skim milk medium - 199, 20% skim milk. ‡ tpb = Tryptose phosphate broth.
§ sm = 100% skim milk.

diluted in TPB medium from that which was incubated and diluted in TPB medium (col. 8 and 9). These last 2 results indicate no evidence for the usual viral inhibitors(11,12).

Discussion. These comparative studies showed that skim milk maintenance medium afforded full sensitivity to the tested strains of polio, echo, coxsackie, influenza, herpes, measles and vaccinia viruses. Parallel titrations of coxsackie A-11 employing skim milk medium and TPB medium with 3 different lots of chicken serum demonstrated higher titers in presence of skim milk. Titer differences noted for adenovirus were not great enough to permit conclusions from 1 experiment; however, consistent differences in 14 experiments became meaningful. Results of further experiments with adenovirus and numerous maintenance media (Table II) revealed that the inhibitory action of the skim milk was probably an effect on tissue culture cells rather than antiviral. Similar cell-mediated effects have been observed by others (9 & 13).

Summary. (1) Comparative titrations of 22 virus strains were performed to determine sensitivity of cell cultures maintained with skim milk medium. Infectivity titers of tested strains of polio, echo, coxsackie, influenza, herpes, measles and vaccinia viruses in presence of skim milk medium were equal to or greater than those employing the established maintenance medium. Lower titers obtained with adenovirus in skim milk medium were attributed to a cell-mediated effect. (2) The results indicate that skim milk medium is free of inhibitor for infectivity and permits full sensitivity to infection by most of the viruses tested.

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Received July 17, 1959. P.S.E.B.M., 1959, v102.

Persistence of Antibodies to ECHO-16 Viruses Following Boston Exanthem Disease.* (25203)

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Of the diseases now considered to be of ECHO virus etiology, Boston Exanthem disease(1,2) was the first to be recognized as a clinical and epidemiologic entity and associated with its causative agent. Immunological relationship of Boston Exanthem viruses to ECHO-16 virus has since been demonstrated, although their antigenic patterns are not identical(3,4). Reports on the ECHO viruses have dealt with clinical associations, epidemiological studies, or antigenic relationships. Information on the immunological status of man several years after infection with ECHO viruses has not been available. Serum samples were obtained† from patients of the 1951 Boston Exanthem disease epidemic, and also from cases of the Pittsburgh outbreak of 1954. This report provides results of studies on complement fixing and neutralizing antibody levels 3 to 6 years after original infection.

Materials and methods. Neutralizing Antibody (NA) Tests. Explant type roller cultures of embryonic human skin-muscle tissue‡, grown with medium containing bovine embryonic fluids as described elsewhere(4), were used. Prior to inoculation the nutrient was changed to 90% bovine embryonic fluids and the Hanks component omitted. Seven days after inoculation one additional fluid change of the same medium was made. Pools of virus in the 4th to 7th *in vitro* passage were used—2 of the *Floyd* strain which originated from a Boston patient, and 2 of the Pittsburgh *McGrew* strain. Serum samples were stored at −15°C, thawed on the day of use, and inactivated at 56°C for 30 minutes. Except as noted in Table I, all serum specimens from a patient were tested simultaneously. Two- or 4-fold serum dilutions were mixed with virus, held at 5°C for 1½ hours, and 3 cultures each were then inoculated with 0.1 ml of the mixture. In each test 3 or more control cultures were inoculated with an equivalent virus dose. Serum NA titers were determined 3 days after the virus controls showed unequivocal and generalized cellular degeneration. Titration of the virus inoculum was performed concurrently with each test. NA titers are expressed as that dilution of serum, before addition of virus, which effected neutralization of 2 or more of the 3 cultures inoculated. If all 3 tubes at one dilution were neutralized and 2 or all 3 tubes of the next highest dilution failed to be neutralized, an arbitrary intermediate dilution was taken as the titer. *Complement-fixation (CF) tests.* Methods of antigen preparation and the micro-method CF test procedure are reported elsewhere(5). The antigens were concentrated from infected culture fluids and heated at 56°C before use. Three antigens, from the same strain (McGrew) of exanthem virus, were used; a few

* This work supported (in part) by grants from Nat. Inst. of Allergy and Infect. Dis., N.I.H., by Lederle Laboratories of Amer. Cyanamid Co., by Parke, Davis and Co., and United Fruit Co.

† Dr. Henry E. Gallup of Dedham, Mass. kindly furnished some specimens from his patients.

‡ We are grateful to Dr. Kurt Benirschke and staff of Boston Lying-In Hospital, and Dr. Harold Rosenfield and staff of Beth Israel Hospital for cooperation in obtaining tissue specimens.

TABLE I. CF and Neutralizing Antibody Titers in Boston Exanthem Disease Patients at Various Intervals after Illness.

Patient, age at onset	Time after onset	CF titer*	Neutralizing antibody titer† *vs* Floyd‡ (Boston) strain	*vs* McGrew§ (Pittsburgh) strain
Mr. W.	20 days	128	64	32
26 yr	2 yr, 2 mo	64	"	16
	4 yr	64	>32	nt
	6 yr, 3 mo	64	nt	128 or >
Mrs. W.	20 days	64	16	2
26 yr	2 yr, 2 mo	32	16	8
	4 yr	16	16	nt
	6 yr, 3 mo	16	nt	8
Lel. W.	23 days	64	32	16
3 yr	2 yr, 2 mo	32	>128	128
	4 yr	16	128 or >	64
	6 yr, 3 mo	16	nt	128
Lin. W.	4 yr	32		128
2 yr	6 yr, 3 mo	32		"
M. F.	11 days	128	64	64
2 yr	2 yr	16	nt	>128
	3 yr, 11 mo	128	>128	256
	6 yr, 3 mo	32	nt	128
V. F.	2 yr, 1 mo	64		128 or >
6 yr	4 yr	128		>128
	6 yr, 3 mo	64		128 or >
C. F.	2 yr, 1 mo	8		128
4 mo	4 yr	16		"
	6 yr, 3 mo	<16		"
Mrs. D.	23-26 days	anticomp.		32 or >
24 yr	6 yr, 3 mo	16		32
L. D.	23-26 days	64		16
2 yr	6 yr, 3 mo	16		32
Mr. McG.	23 days	128	16	32
29 yr	11 mo	64	"	"
	2 yr, 10 mo	32	"	"
D. McG.	27 days	128	64	128
4 yr	11 mo	32	>64	256
	2 yr, 10 mo	16	"	128
R. We.	20 days	128		32
5 yr	11 mo	32		64
	2 yr, 10 mo	16		>32
K. We.	20 days	64		32 or >
6 yr	11 mo	16		64
	2 yr, 10 mo	16		32 or >
Mrs. We.	17 days	64		128
30 yr	11 mo	16		"
	2 yr, 10 mo	8		16
Mr. Du.	18 days	256		64
35 yr	11 mo	64		<32
	2 yr, 10 mo	32		32

* All titers expressed as reciprocal of serum dilution before virus or antigen added.

† Neutralized for 3 days after virus control cultures showed degeneration.

‡ Virus dose varied from 20 to 350 ID_{50}.

§ *Idem* 25 to 250 " .

nt = not tested.

tests were done with an antigen derived from another strain (Clauss). Two units of antigen and 2½ to 3½ units of complement were employed. A positive control serum was included in each test and reacted to comparable degree with each antigen used(5). Available serum samples from any one patient were tested simultaneously at least once. Duplicate tests showed good agreement in titers.

Results. The level of CF antibody usually showed a 2- to 4-fold decline in titer within one year after infection, but little change thereafter for as long as 6 years. One Boston patient (M. Flo) exhibited a distinct secondary rise in CF antibody titer between the second and fourth years. However, no significant increase in level of NA was evident over this same interval. CF and NA values for the 15 patients studied are summarized in Table I.

With one possible exception, no decline in NA level was found 3 to 6 years after the original infection. However, unexplained fluctuations in titer of NA were recorded for sera obtained from one family (Will.). This variation was not associated with corresponding changes in CF titer, and in only one patient (Lel. Will.) was the fluctuation in NA titer demonstrable with both test strains of virus.

Discussion. The results indicate that in man after infection with ECHO 16-like viruses, CF antibody titers fall moderately within 1 to 2 years, and then persist at detectable levels for periods of at least 3 to 6 years. This contrasts with the more rapid drop in CF antibody levels following infection with polio viruses(6). Evidence was also obtained that a secondary rise in CF antibody may occur, presumably because of reinfection with the same or an antigenically related agent.

Levels of NA showed no significant decline 3 to 6 years after the exanthem infection; in fact, apparent increases in titer were registered in several cases. However, the significance of these changes in late convalescence is difficult to evaluate. The individuals with unstable antibody levels were all members of the same family. In 2 instances the rise in titer may have represented a gradual increase in antibody after the original infection, since

no samples were tested between early convalescence and 2 years. The absence of a concomitant rise in CF antibody would support this hypothesis. If the changes in antibody levels were the result of infection with antigenically related viruses, a discrepancy in booster antibody response between neutralizing and CF antibodies would have to be postulated.

It is possible that the levels of antibody in the patients studied were maintained by inapparent infections with antigenically related enteroviruses. Detection of changes in antibody titer resulting from such a mechanism would require more frequent sampling of sera. Further investigation will be necessary to determine whether the pattern of immunologic response found in Boston Exanthem disease is also applicable to other ECHO virus infections.

Summary. Persistence of complement fixing and neutralizing antibodies to ECHO 16-like viruses was studied in 15 patients convalescent from Boston Exanthem disease. CF titers fell moderately within 1 or 2 years after infection, but remained demonstrable for 3 to 6 years. Levels of neutralizing antibody persisted without significant decline for a similar period of time.

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Received July 23, 1959. P.S.E.B.M., 1959, v102.

Nucleic Acid Content of Various Areas of the Rat Brain.* (25204)

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The present investigation was initiated to provide control values of NA concentrations in local areas of rat brain. Previous reports have, for the most part, concerned themselves with NA content of the whole brain ignoring the differences between the various areas of the brain. Since these areas differ from each other functionally, morphologically, metabolically, and in chemical composition, and since physiological stresses or agents are known to act differently on these various cell groups, it becomes necessary to establish regional control values.

Methods. Adult, white, male rats (300-450 g) were used. The rats were decapitated after receiving a stunning blow on the head and the brain dissected on a cold surface. The tissue was washed with ice-cold distilled water to remove surface blood and blotted on filter paper. More reproducible results were obtained if the tissue was blotted to remove surface water (Table I). The excised tissue was then placed in 10% trichloracetic acid (TCA) or stored in the deep-freeze. A maximum period of 15 minutes elapsed between sacrificing of the animal and the storage or treatment of the tissue with TCA. When tissue weight was 100-200 mg, the homogenates were prepared with a close fitting stainless steel pestle in 16 × 150 mm test tubes. When the hypothalamic and thalamic tissues (20-50 mg) were analyzed for total nucleic acid (TNA), the tissue was homogenized in a 12 ml centrifuge tube using a plastic pestle prepared according to the method of Darbé(1). It was necessary to pool these tissues from 5 animals to obtain enough material for analysis of deoxyribonucleic acid (DNA) using the standard procedure. Enzymatic assays were made following the procedure of McDonald(2) except that it was run at 37°C and different

* This work was partially supported by grant from Nat. Inst. of Mental Health and by Contract between U. S. Air Force and Univ. of Md.

TABLE I. Nucleic Acid Contents of Different Areas of Rat Brain.

Area	Preparation	mg P/100 g wet wt TNA	DNA	RNA	RNA/DNA
Cerebral cortex	Frozen (unblotted)	21.2 ± 7.1 (7)*	6.9 ± 1.0 (8)	14.3 ± 7.2	2.1 ±1.1
	Frozen (blotted)	22.1 ± 2.1 (9)	7.5 ± .1 (6)	15.6 ± 2.3	2.0 ± .3
	Fresh	22.5 ± 1.3 (6)	7.0 ± .6 "	15.5 ± 1.5	2.1 ± .3
White matter	Frozen	17.0 ± 1.4 (8)	8.8 ± 2.3 (7)	8.2 ± 2.7	.93 ± .4
	Fresh	18.4 ± 2.7 (6)	8.4 ± 1.1 (8)	10.0 ± 2.9	1.2 ± .4
Cerebellum (whole)	Frozen	50.0 ± 1.5 "	39.4 ± 4.5 (7)	10.6 ± 4.7	.27 ± .12
	Fresh	58.3 ± 2.8 "	38.2 ± 4.0 (8)	20.1 ± 4.9	.53 ± .14
Hypothalamus	Frozen	18.2 ± 3.6 (4)		7.9 ± 4.3	.77 ± .46
	Fresh	29.8 ± 5.1 (8)	10.3 ± 2.4 (3)	19.5 ± 5.6	1.9 ± .7
Thalamus	Fresh	20.4 ± 2.3 "	8.3 ± 1.1 "	12.1 ± 2.6	1.5 ± .4
Medulla	Frozen	13.9 ± 2.3 (6)			
	Fresh	13.0 ± 1.7 "	9.4 ± 2.4(14)	3.6 ± 2.9	.38 ± .3

* Mean ± stand. dev. No. in parentheses is No. of determinations.

time intervals were used. RNA and 5′ adenylic acid were used as substrates to measure the activities of ribonuclease (RNase) and 5′ nucleotidase respectively. TNA and DNA were determined by using the ultraviolet absorption method of Logan *et al.*(3) and RNA content was calculated as the difference between TNA and DNA. The value of the absorptivity, a_p, at 268 mμ used in this investigation was 320 expressed in terms of mg NAP(3). The atomic extinction coefficient, $\epsilon(P)$, is 30.98 times a_p or 9814. All phosphorous analyses were made using the Fiske-Subbarow method(4).

Results. In the course of determining DNA, the residue from the lipide extraction is treated with KOH to hydrolyse RNA. A residue was inevitably found after hydrolysis. The only previous report of a residue is that found during the analysis of bone(5). The quantity of material which absorbed at 260 mμ using perchloric acid extracts of the residue(6) amounted to approximately 0.5% of the DNAP in the white matter and cerebellum.

Handling of the tissue after removal from the brain affects the NA concentrations to different extents in different areas of the brain (Table I). More variable results were obtained when the tissue was not blotted after washing with ice-cold water to remove surface blood. Samples of cerebellum and hypothalamus which had been stored in the deep-freeze had significantly lower TNA contents than tissue analysed immediately after the animal had been sacrificed ($p < 0.01$). The differences in the other areas of the brain were not statistically significant ($p = 0.10$). DNA analyses in all areas gave the same values whether the tissue was fresh or frozen. RNA content was reduced by 47% in the cerebellum and by 60% in the hypothalamus as a result of storage in the deep-freeze. If the tissue was dissected without being on a cold block, TNA was reduced by 20% in the cortex, 27% in white matter, and 37% in the cerebellum.

Several preliminary experiments were performed to determine whether enzymes which act on RNA were present to a greater extent in the cerebellum than the cerebral cortex. Extracts of these tissues prepared with phosphate buffer, pH 7.3, were incubated overnight with RNA. Changes in absorbance at 260 mμ were 2.5 times greater with cerebellum than with cerebral cortex per g tissue. Additional studies were performed with tissue homogenates at different time intervals and using RNA and AA as substrates. The results are given in Table II. About 17% per organic P was liberated by the action of cerebellar homogenates on RNA than with cortical homogenates although changes in absorbance at 268 mμ were greater with cortical homogenates than with cerebellar homogenates after 2 hours. Cerebellar homogenates liberated 44% more inorganic P than cortical homogenates with AA as the substrate.

TABLE II. Enzyme Studies on Rat Cortex and Cerebellum Homogenates.

Substrate	Ribonucleic acid						5′ adenylic acid	
Tissue	Cortex			Cerebellum			Cortex	Cerebellum
Measurement	A_{268}†	Inorg. P*	Organic P*	A_{268}†	Inorg. P*	Organic P*	Inorganic P*	
Time, min.								
10	.03	.02	.08	.00	.05	.07	.04	.04
30	.08	.05	.24	.02	.14	—	.17	.14
120	.25	.25	.98	.12	.20	1.15	.39	.56

Values represent change from t = 0. Solutions containing 1 ml homogenate (40-60 mg tissue/ml acetate buffer, pH 5.0) and 1 ml substrate (0.5 mg RNAP or 0.5 mg 5′ adenylic acid/ml buffer) incubated at 37°C.

* μg P/mg wet wt. † $\triangle$A/mg wet wt.

Discussion. The results of NA analyses (Table I) in the various areas of the brain indicate that fresh tissue should be used for TNA analyses. However, it is not necessary to use fresh tissues when DNA concentrations are being measured. The differences between RNA in fresh and frozen samples of cerebral cortex and cerebellum might be explained by either of the 2 following hypotheses: 1) A labile RNA is present in cerebellum which is degraded during the slow freezing and thawing processes or is present to a greater extent in the cerebellum than in the cerebral cortex. Smith(7) found that using dry ice instead of cracked ice during homogenization of tissues increased TNA concentration by 18%. However, the analysis was always the same in frozen liver as in fresh tissue. 2) An enzyme or enzymes are present in greater concentration in the cerebellum than in the cerebral cortex, which degrades RNA during the freezing and thawing process.

There is less RNase activity and more 5′ nucleotidase activity in the cerebellum than in the cerebral cortex (Table II). Similar results have been reported for distribution of RNase in rabbit brain(8) and human brain(9) and for 5′ nucleotidase in rat brain(10). Hence, RNA in the cerebellum may be degraded more rapidly than in the cerebral cortex because of the large concentration of this latter enzyme which acts not upon RNA but on its degradation products. However, the true concentrations of RNase in the various areas may be different from the values found with the assay conditions used in this investigation.

The NA contents of rat cerebral cortex previously reported have been higher than those reported in Table I. These analyses(11) used color tests which have been shown to yield higher results than the ultraviolet absorption method(3).

The amount of DNA is approximately the same in all areas except the cerebellum (Table I). Similar observations have been reported for rabbit(8) and cat(12). Microdeterminations on rabbit and monkey cerebellum have also been reported by Kissane and Robbins (13). However, distribution of RNA is different in rat brain with highest concentrations found in the cerebellum, hypothalamus, and cerebral cortex (Table I). The lowest amount of RNA is in the medulla. Similar results have been found with cat tissues(12).

It is interesting to note that protein content is high in the areas where RNA concentration is high (cerebral cortex, cerebellum, and hypothalamus), but the content of both substances is low in medulla(14).

A knowledge of DNA concentration permits an attempt at calculation of tissue components such as average cell densities, dry weight/average cell, and total number of cells in each brain area (Table III). These calculations are based upon the assumption that amount of DNA/cell is constant in all cerebral cells (see discussion of Nurnberger and Gordon (15)). Although no direct measurements of DNAP/cell have been reported for rat brain, an average value of 65×10^{-8} μg DNAP/nucleus is used in these calculations(16). Comparison of the calculated cell densities from chemical analyses and those obtained by direct measurement(15) indicates close agreement between these two different methods. The difference is less than 25% in most areas, but is 40% for white matter. This disagreement may be due to different definitions of

TABLE III. Cell Densities, Weight/Cell, and Total Number of Cells in Areas of Rat Brain.*

Analyte	Area					
	Cortex	White matter	Cerebellum	Hypothalamus	Thalamus	Medulla
DNAP, μg/g wet wt	70 ± 6§	84 ± 11	382 ± 40	103 ± 24	83 ± 24	94 ± 24
Cell density, cells/g wet wt × 10^8	1.08 ± .09	1.29 ± .17	5.88 ± .62	1.58 ± .37	1.28 ± .17	1.45 ± .37
Nurnberger & Gordon (15)	.96	.78	4.77	1.79	1.21	1.13
Wet wt/cell, 10^8 pg/cell	9.3	7.8	1.7	6.3	7.8	6.9
% moisture	78.8†	80.0‡	78.4†	80.0‡	80.0‡	77.1†
Dry wt/cell, 10^8 pg/cell	2.0	1.6	.37	1.3	1.6	1.6
Wet wt, mg	400	175	250	40	40	100
Total No. of cells/area, 10^7	4.3	2.3	14.7	.63	.51	1.5

* pg = 10^{-12}g cell density = $\frac{\mu\text{g DNAP/g wet wt}}{65 \times 10^{-8}\ \mu\text{g DNAP/nucleus (cell)}}$. Wet wt/cell = 1/cell density. Dry wt/cell = (100 − % moisture) × wet wt/cell. Total No. of cells/area = Cell density × wet wt of area.

† Gosselin *et al.*(17). ‡ Assumed value. § Mean ± stand. dev.

the areas or difficulties in selection of pure white matter. It should be noted that all values in Table III represent the composition of the average cell and do not distinguish between neuronal and supporting cells. However, this type of calculation can supply valuable information about changes in composition of the cell produced by altering the physiological state of the tissue.

Summary and Conclusions. Nucleic acid analyses were made on the various areas of the rat brain using the ultraviolet absorption method. It was found necessary to use fresh tissues to obtain correct ribonucleic acid concentrations since analyses in some areas (cerebellum and hypothalamus) were lower when the tissues had been kept frozen. Enzymatic analysis indicated that the higher concentration of 5′ nucleotidase in cerebellum than in cortex might be indirectly responsible for the loss of the ribonucleic acid during the freezing and thawing processes. Deoxyribonucleic acid concentration in the cerebellum is approximately 4-5 times the amount found in the other areas. Ribonucleic acid content of the brain decreased in the order: cerebellum, hypothalamus, cerebral cortex, thalamus, white matter and medulla. Average cell densities, weight/cell, and total cells/area were calculated from the deoxyribonucleic acid concentrations. Close agreement between the calculated cell densities and direct measurements were found.

The authors wish to thank Jane Baatz, Nancy H. Eidman, Carole S. Rubin, and Epp Tammaru for their technical assistance.

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Received March 19, 1959. P.S.E.B.M., 1959, v102.

Metabolic Alteration of 2-Methylthio-4-Amino-5-Hydroxymethylpyrimidine (Methioprim).*† (25205)

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Amethopterin-resistant mutants of *Bacillus subtilis* and mutants of *Escherichia coli* resistant to purine antagonists show increased sensitivity to the inhibitory effect of methioprim (2 - methylthio - 4-amino-5-hydroxymethylpyrimidine) when compared to the parent organisms, *i.e.,* collateral sensitivity(1). Significant growth suppression of subcutaneous tumors (Ehrlich carcinoma, clone 2 and Krebs) is also observed with this compound(2). Furthermore, chromatography of urine from dogs and rats receiving methioprim reveals, in both cases, the excretion of an additional ultraviolet-absorbing substance with anti-bacterial activity similar to methioprim‡. The present study reports that rat liver readily metabolizes methioprim to at least 3 products.

Materials and methods. Rat liver incubations. Adult male Sprague-Dawley rats were sacrificed by decapitation. Livers were immediately excised and placed in chilled bath of Krebs phosphate buffer, pH 7.4(3). Slices weighing 70-100 mg were cut with a Stadie-Riggs microtome. Three slices were placed in each 20 ml beaker with 3 ml of Krebs buffer solution to which methioprim (20 μmoles/ml) was added. The buffer-substrate mixtures and control beakers were incubated under atmosphere of 95% O_2-5% CO_2 at 36.5°C with constant shaking in Dubnoff metabolic incubator. At end of 5 hours, the reaction was stopped and protein precipitated by rapidly heating in boiling water bath for 5 minutes followed by cooling. The supernatant fluid was collected by centrifugation for analysis of metabolic products. Aliquots of supernatants of incubation mixtures and controls were applied to large sheets of Whatman 3 MM paper and ascending chromatograms were developed. Initial separation of components of incubation mixtures was carried out using top layer of solvent prepared by mixing n-butyl alcohol (500 ml): water (500 ml); glacial acetic acid (16 ml). Chromatograms were examined for metabolic products by using ultraviolet light, and various spray reagents and by bioautographic technic using microbiological assay described below. *Microbiological assay.* Methioprim and some closely related compounds suppress growth of amethopterin-resistant mutants of *Bacillus subtilis* to a greater extent than the parent strain(1). Such compounds have no effect on growth of these strains when 2 - methyl-4-amino-5-aminomethylpyrimidine (the pyrimidine moiety of thiamine) is present in growth medium. For routine purposes, an agar-diffusion method and a chemically-defined medium(4) were used. An amethopterin-resistant mutant of *Bacillus subtilis,* designated as strain 6051/A, was homogeneously seeded into medium with and without a supplement of 2-methyl-4-amino-5-aminomethylpyrimidine. Similar platings of the parent *B. subtilis* 6051 were also made. Either paper discs impregnated with test solutions or strips of paper chromatograms were placed on the

* This investigation was supported, in part, by research grant from Nat. Cancer Inst., U.S.P.H.S.

† A preliminary report was presented at annual meeting of Amer. Assn. for Cancer Research, Chicago, Apr. 12, 1957.

‡ R. Guthrie, unpublished data.

surface of assay plates and incubated at 25°C up to 3 days. Zones of inhibition were noted. Presence of methioprim (concentrations as low as 10^{-7} M can be detected) or similar compounds may be ascertained easily by characteristics described above. *Chemicals.* 2-Methylthio-4-amino-5-formylpyrimidine was prepared by the method of Okuda and Price (5), 2-thio-4-amino-5-pyrimidinecarboxylic acid and 2-methylthio-4-amino-5-pyrimidinecarboxylic acid by the method of Ulbricht and Price(6). 2-Methylthio-4-hydroxy-5-hydroxymethylpyrimidine was prepared by reduction of corresponding carbethoxypyrimidine with lithium aluminum hydride in ether. The yield in this solvent was low; however, recently, Hoover(7) reported synthesis of this compound in good yield by use of dimethyl ether of diethyleneglycol as solvent. 2-Methylsulfonyl-4-amino-5-formylpyrimidine was prepared by the method of Nairn(8). 2-Thio-4-amino-5-hydroxymethylpyrimidine and methioprim were kindly supplied by Dr. Stanton Harris and Dr. James Sprague, Merck and Co. The other compounds were obtained from commercial sources.

Results. Initial chromatograms of incubation mixtures revealed 4 new components designated as follows according to behavior under ultraviolet light: Component I, Rf 0.95—fluorescent; Component II, Rf 0.82—ultraviolet-absorbing; Component III, Rf 0.75—fluorescent; and Component IV, Rf 0.25—ultraviolet-absorbing. Components I, II and IV inhibited the assay organism *B. subtilis* 6051/A. When amounts of methioprim equivalent to incubation concentrations were chromatographed as a control, an ultraviolet-absorbing contaminant was observed with the same Rf value as Component IV. The nature of this compound will be discussed below. As measured microbiologically 25-30% of methioprim was metabolized.

Isolation and identification of metabolic products. Component I: This compound appears in low yield and has not been identified. It does not react with ninhydrin or dihydropyrimidine spray reagents. Its Rf value and/or ultraviolet spectrum differs from all the following compounds: 2-thio-4-amino-5-pyrimidinecarboxylic acid, 2-methylthio-4-amino-pyrimidine, 2-methylthio-4-amino-5-methylpyrimidine, 2-thio-4-amino-5-hydroxymethylpyrimidine, 2-methylsulfonyl-4-amino-5-formylpyrimidine, 2-methylthio-4-hydroxy-5-hydroxymethylpyrimidine, uracil, 5-hydroxymethyluracil, cytosine, and 5-hydroxymethylcytosine.

TABLE I. Comparison of Component II with Synthetic 2-Methylthio-4-amino-5-formylpyrimidine.

	Synthetic compound	Product isolated from incubations
Sulfur	Positive	Positive
Melting point	192 uncorr	192-93 uncorr
Nitrogen	Positive	Positive
M.P. of semicarbazone	272 uncorr	270-72 uncorr
Infrared spectrum and ultraviolet spectrum	In both cases no discernible difference	
Molar extinction coeff.	Both 1.14×10^4 at 316 mμ	
Rf values†	In both cases no discernible difference	
Microbiol. assay*	Inhibitory*	Inhibitory*

* Microbiological assay: Inhibits *B. subtilis* 6051/A. Growth inhibition reversed by 2-methyl-4-amino-5-aminomethylpyrimidine.

† Solvents (see text for ratios)

1. n-butyl alcohol—glacial acetic acid-water — Rf = 0.82
2. ethanol—NH_4OH — Rf = 0.79
3. n-propyl alcohol—water — Rf = 0.85

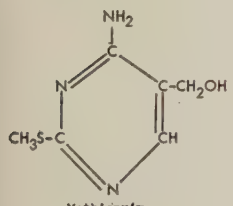
Component II: This component was eluted from initial chromatograms with 95% ethanol and purified further by successive ascending chromatography in ethanol(1): 1N NH_4OH (1), and then n-propyl alcohol(1): water(1). Purified Component II was recrystallized from absolute ethanol. On the basis of series of tests listed in Table I, it was concluded that Component II was identical to 2-methylthio-4-amino-5-formylpyrimidine.

Component III: Isolation of this compound was achieved with identical procedure used for component II. Table II shows comparative tests made with 2-methylthio-4-amino-5-pyrimidinecarboxylic acid. It was concluded that component III and 2-methylthio-4-amino-5-pyrimidinecarboxylic acid were identical.

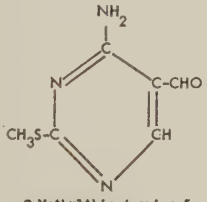
Component IV: This compound was also eluted from initial chromatograms with 95% ethanol and purified by successive ascending chromatography with n-butyl alcohol saturated with water, and then n-propyl alcohol

(6): HCl(2): water(2). The properties of this material are listed in Table III. Several stock samples of methioprim contained a small concentration of an ultraviolet-absorbing contaminant with an Rf value similar to Component IV. After reviewing the method for preparation of methioprim(6) it was shown that the contaminant was formed in the reduction of corresponding carbethoxypyrimidine to methioprim with lithimum aluminum hydride. Purification of this yellow-colored, toluene-insoluble substance by chromatography on a cellulose column eluted using top layer of a mixture of n-butyl alcohol (500 ml) : glacial acetic acid (16 ml) : water (500 ml) and analysis has led to the conclusion that it is an aluminum containing derivative of methioprim arising from incomplete hydrolysis of the reduction intermediate. The properties of this material are also listed in Table III.

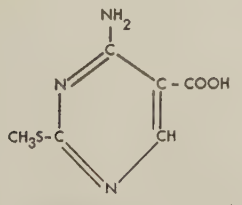
Discussion. The present data demonstrate that the metabolism of methioprim by rat liver slices involves an oxidation of the 5-hydroxymethyl group to 5-formyl and 5-carboxylic acid derivatives as well as an unidentified ultraviolet-absorbing material. This sequence is shown in the following diagram:



Methioprim



2-Methylthio-4-amino-5-formylpyrimidine



2-Methylthio-4-amino-5-pyrimidine-carboxylic acid

Despite precise knowledge as to the nature of the unidentified product, it does not appear to be a decarboxylated or deaminated derivative of 2-methylthio-4-amino-5-pyrimidinecarboxylic acid.

The catabolism in the rat of nucleic acid pyrimidines, uracil and thymine, has been shown by several investigators(9,10,11) to proceed *via* reduction to corresponding dihydropyrimidines prior to ring cleavage yielding *beta*-alanine and *beta*-aminoisobutyric acid respectively. Existence of an oxidative mechanism was substantiated by the observation that incubation of radiothymine with rat liver slices also gave rise to 5-hydroxymethyluracil and uracil-5-carboxylic acid(12). The oxidative metabolism of uracil and thymine in microorganisms has been reported in a soil bacterium to involve only a limited attack of the 6-C

TABLE II. Comparison of Component III with Synthetic 2-Methylthio-4-amino-5-pyrimidinecarboxylic acid.

	Synthetic compound	Product isolated from incubations
Sulfur	Positive	Positive
Nitrogen	"	"
Melting point	220-30 dec.‡	219-228 dec.‡
Ultraviolet spectrum	In both cases no discernible difference	
Ultraviolet nature	Fluorescent	Fluorescent
Rf values†	In both cases no discernible difference	
Microbiological assay*	Negative	Negative

* Microbiological assay: No inhibition of *B. subtilis* 6051 or *B. subtilis* 6051/A.

† Solvents (see text for ratios)

1. n-butyl alcohol—glacial acetic acid-water Rf = 0.75
2. ethanol—NH_4OH Rf = 0.32
3. n-propyl alcohol—water Rf = 0.60

‡ Uncorr.

TABLE III. Comparison of Component IV with Impurity Isolated from Stock 2-Methylthio-4-amino-5-hydroxymethylpyrimidine.

	Synthetic compound	Product isolated from incubations
Sulfur	Positive	Positive
Lithium	Negative	Negative
Aluminum	Positive	Not determined
M. P.	200-210 dec uncorr	200-210 dec uncorr
Acidity and basicity	Amphoteric	Amphoteric
Ultraviolet absorption		
.1N HCl	Max 245-250 mμ	Max 245-250 mμ
.1N NaOH	Max 284 mμ	Max 284 mμ
Treatment with warm acid	Yields methioprim	Not determined
Physical nature	Hygroscopic	Not isolated as a solid

position of the pyrimidine ring(13). Others (14,15) have obtained barbituric acid from uracil oxidation by strains of Nocardia, Corynebacteria, and Mycobacteria.

The biological oxidation of the hydroxymethyl group to an acid is not limited to pyrimidines. Pyridoxine is converted to 4-pyridoxic acid(16) and other data implies that pantothenyl alcohol gives rise to pantothenic acid (17).

It is of interest to note that the magnitude of antibacterial activity as measured in this study declines in sequence with the oxidation of methioprim *i.e.* $-CH_2OH > -CHO > COOH$ (inactive).

Summary. Rat liver slices convert methioprim to 2-methylthio-4-amino-5-formylpyrimidine and 2-methylthio-4-amino-5-pyrimidinecarboxylic acid. Another unidentified ultraviolet-absorbing material is also produced. The antibacterial activity of methioprim is greater than the 5-CHO derivative, whereas the 5-COOH derivative is inactive.

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Received June 22, 1959. P.S.E.B.M., 1959, v102.

Ascending Infection as a Mechanism in Pathogenesis of Experimental Non-Obstructive Pyelonephritis.* (25206)

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Experimental pyelonephritis has generally been produced by methods involving intra- or extra-renal obstruction to the flow of urine with subsequent injection of bacteria into the blood stream. Ureteral ligation(1,2), electrocauterization(3), renal massage(4), or the scars of antecedent staphylococcal infection (5) have resulted in the localization of bacteria in the traumatized kidney with subsequent development of acute pyelonephritis. In addition, *S. aureus* in rabbits and mice(6), *P. aeruginosa* in mice(7), *S. fecalis* in rats (8) and *C. renale* in mice(9) have, after intravenous injection, produced infections of kidney in the absence of induced urinary obstruction. The experimental models involving obstruction and hematogenous dissemination have been widely studied. In the absence of a clear demonstration that infection of the kidney may ascend directly from the bladder, the concept has become increasingly accepted that the hematogenous route is the principal one for the spread of infection to the kidneys, although many investigators have indicated the lack of definitive experimental informa-

* Aided by grants from Nat. Inst. of Health, U.S.P.H.S., and Massachusetts Heart Assn.

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tion(10). Recently, however, it has been shown that asymptomatic bacteriuria in man not only may be associated with unsuspected pyelonephritis, but also may precede development of pyelonephritis(11-14). These observations suggested that production of persistent bacteriuria might be followed by infection of the kidneys. The present studies demonstrate that when bacteriuria is produced in the urinary bladders of rats, ascending pyelonephritis occurs regularly in the absence of demonstrable obstruction.

Materials and methods. The test organism was a strain of *Proteus vulgaris* grown overnight in nutrient broth, chosen because it was the most pathogenic to rats of a large number of strains of gram negative rods studied. Bacteria were injected directly into the bladder after the abdomen had been opened. When glass beads were used, they were inserted through an incision in the dome of the bladder and the bacterial culture injected immediately afterward. The defect in the bladder was closed by double ligatures. The experimental animals were male white rats weighing 150-200 g; they were sacrificed using ether anesthesia and the kidneys removed aseptically through separate flank incisions. The kidneys were homogenized in sterile broth using glass homogenizers. Serial dilutions of the homogenate, in nutrient broth, were transferred to agar pour plates for colony counts. Heart's blood was obtained antemortem by percutaneous cardiac puncture and urine for culture was obtained by aspiration through the bladder wall after the abdomen had been opened aseptically.

Results. Preliminary experiments showed that when about 10^8 bacteria were introduced into urinary bladders of rats, 60% of the animals developed anatomic and bacteriologic evidence of pyelonephritis within 4 days. If, in addition, a glass bead was inserted into the bladder lumen, pyelonephritis occurred in 95% of the animals within 4 days. The infection was bilateral in 97% of animals. When 10^9 bacteria were injected intracardially, 15% of the animals had pyelonephritis within 4 days; when a glass bead was in the bladder, incidence of infection of the kidney after hematogeneous dissemination of the bacteria rose to 40%. In general, when there were morphologic lesions of active pyelonephritis, colony counts of the kidney were greater than 10^6.

When about 10^8 bacteria were inserted into the bladders without glass beads, the bacteria were found in 55-60% kidneys, 24 hours later (Table I). When one ureter was doubly ligated, sectioned between ligatures, and bacteria then inserted into the bladder, bacteria were found in 50% of kidneys with uninterrupted ureters, but were present in only 9% of kidneys with sectioned ureters. When the experiment was performed with a glass bead in the bladder, bacteria were found in 75% of kidneys on the intact side, and in 35% of kidneys on the interrupted side. In almost every instance in which bacteria were found in the kidneys with ligated ureters, the blood cul-

TABLE I. Effect of Ureteral Section on Bacterial Colony Counts in Kidneys of Rats after Administration of *Proteus vulgaris.*

Site of inj.	Bead in bladder	No. rats	Kidney	Bacterial colony count in kidney at 24 hr			
				$>10^6$	10^{4-6}	10^{0-3}	0
Bladder; ureters intact	No	29	Right	34%	0%	25%	41%
			Left	34	3	18	45
" left ureter sectioned	"	34	Right	26	12	12	50
			Left	0	0	9	91
" ureters intact	Yes	20	Right	70	0	0	30
			Left	70	0	10	20
" left ureter sectioned	"	28	Right	61	11	3	25
			Left	3	7	25	65
Heart; left ureter sectioned	No	21	Right	10	24	57	9
			Left	48	5	42	5
Idem	Yes	27	Right	4	4	33	59
			Left	30	7	26	37

tures were positive for Proteus.

The results are not due to greater susceptibility of the uninterrupted side to hematogenous infection; when 10^{6-9} bacteria were given intracardially, the kidneys with obstructed ureters were more susceptible to hematogenous infection than were the intact kidneys (Table I).

Discussion. The experiments cited here leave little doubt that, after induction of bacteriuria, ascending infection of the kidneys may occur. Whether this pathway is the most common one in man remains to be demonstrated, but there is strong circumstantial evidence to suggest that bacteriuria in man leads to pyelonephritis and that the most probable route is an ascending one(10,13,15).

Whether the ascension of infections in rats is by way of multiplication and diffusion of bacteria in the lumen containing urine, or is by way of tissue spaces and lymphatics remains to be seen.

The observations are consistent with the earlier observations of David(16). The reasons why earlier workers failed to establish bacteriuria experimentally are not clear. It is likely, on the basis of preliminary observations in this laboratory, that the ease with which persistent bacteriuria was produced was related to selection of a strain of bacteria that was highly virulent for the experimental animals. All too frequently, attempts to produce experimental pyelonephritis have involved the use of bacterial strains that were pathogenic to man and were but weakly pathogenic to the experimental animals used.

Summary. 1. When bacteriuria is induced in rats by instillation of *P. vulgaris* into the urinary bladder, pyelonephritis is produced consistently in the absence of demonstrable obstruction of the urinary tract. 2. Ligation and section of one ureter greatly impairs spread of bacteria to the kidney on affected side. Kidneys with ligated ureters are, however, more susceptible than unaffected kidneys to hematogenous spread of the organisms. 3. Thus, bacteriuria, as produced here, leads to ascending infection of the kidney in absence of demonstrable abnormalities of the urinary tract.

We are indebted to Carole Sheft for valuable assistance.

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Received July 9, 1959. P.S.E.B.M., 1959, v102.

Human Metabolism of Orally Ingested Glycyrrhetinic Acid and Monoammonium Glycyrrhizinate.*† (25207)

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Triterpene glycyrrhetinic acid (G.A.) and the ammonium salt of its diglucuronide, monoammonium glycyrrhizinate (MAG) have been studied in view of their reported adrenocortical-like activity effects on electrolyte metabolism(1,2,3,4). Other authors found G.A. had potential anti-inflammatory properties(5,6,7) and blocked *in vitro* the metabolism of certain steroids(8). Since there are no known chemical methods of determining G.A. or its metabolites, and since we have previously shown (9) that totally adrenalectomized human subjects, maintained solely on MAG, excreted significant amounts of steroid-like materials in urine, as indicated by formation of Porter Silber (phenylhydrazone) chromogens, we studied the *in vivo* metabolism of G.A. and MAG randomly labelled with tritium.

Methods, apparatus and materials. Both MAG and G.A.‡ were labeled with tritium by the New England Nuclear Corp., following which they were recrystallized to constant specific activity (15.7 μc/mg and 63.6 μc/mg respectively. All countings were done using a Packard Tri-Carb liquid scintillation counter. Complete urine collections from subjects receiving labeled compounds were pooled, concentrated *in vacuo* to about 1/20th original volume, and extracted with ethyl acetate to give the "free" fraction. Urines were then brought to pH 11 and exhaustively extracted with n-butanol to remove the "conjugates." Butanol was removed *in vacuo*, the residue taken up in pH 4.5 acetate buffer and incubated with beta-glucuronidase (Ketodase, Warner-Chilcott), then extraction with ethyl acetate was repeated to remove materials formerly present as glucuronic-acid conjugates. Residual aqueous phase was brought to pH 0.4 with sulfuric acid and allowed to stand 2 days at 37°C to bring about further hydrolysis, and similarly extracted. Radioassay of aliquots of all organic extracts, as well as all aqueous phases was carried out. Fecal samples were extracted twice by homogenizing with alcohol in a Waring Blendor, and filtered. (Soxhlet extraction of dry residue remaining after second hemogenization gave negligible additional activity.) The column chromatographic system used, both on urinary and fecal extracts, was a modification of that described by Van Katwijk and Huis in't Veld (10), in which the crude material is placed on a florisil column in benzene solution and eluted with increasing concentrations of alcohol in benzene. In all cases, when counting aqueous samples (blood and bile) or highly colored extracts of organic solvents (from urine and feces), known amounts of tritium standard were added to the samples after counting to obtain a correction for the quenching caused by color and/or solvent.

Results. Values of urinary excretion of radioactivity following oral administration of labeled MAG and G.A. to 4 human subjects are shown in Table I. Urines of female subjects, subsequently concentrated and used for isolation studies, showed the same degree of activity both before and after concentration, thus eliminating the possibility of large-scale formation of H^3OH which would have been lost in the distillate upon concentration. Excretion of a very minute percentage of administered radioactivity in urines was confirmed by results with normal male subjects, and, in addition, presence of practically all radioactivity in feces within 48 hours following ingestion of tagged compounds was conclusively demonstrated. Purification of the fecal extract of the male subject,

* This work supported by grants from U.S.P.H.S., and from G. D. Searle & Co., Chicago, Ill.

† Presented at meeting of Fed. Biol. Soc., 1959, v18, 349.

‡ Part of MAG and G. A. were supplied through kindness of Dr. Willard Hoehn, G. D. Searle & Co.

TABLE I. Metabolic Fate of H^3-Labeled MAG and G.A.

Age	Sex	Diagnosis	Amt/day MAG	Amt/day G.A.	Duration, days	% adm. activity found in Urine	% adm. activity found in Feces
48	♀	Breast cancer—adrenalectomized	*1 mC H^3		5	.3	
50	♀	11 yr Addison's disease		†1 mC H^3	5	.4	
35	♂	Normal	*1 mC H^3		1	.8	98
40	♂	"		†2 mC H^3	1	.4	"

* Plus 4 g carrier. † Plus 3 g carrier.

fed G.A., resulted in isolation of a small quantity of radioactive, crystalline material whose infrared spectrum was the same as that of the administered compound (Fig. 1). This shows that at least a portion of G.A. is excreted unchanged in the feces. A complete characterization of all radioactive fractions has not yet been accomplished.

The next problem was to determine whether MAG and G.A., under these conditions, pass directly through the GI tract without being absorbed (except to a very small degree) or whether they are absorbed into the blood and later excreted via the bile. To answer this question, 5 normal human subjects were fed the labeled compounds and radioassays were performed on simultaneously collected blood and urine samples, and, in 2 of these subjects, on bile. The results (Table II) show that values are all 1% or less of total dose, so that absorption into the blood and re-excretion via the bile may be dismissed as a major metabolic pathway of these ingested materials.

Finally, we were able to isolate and identify by infrared spectroscopy pure glycyrrhetinic acid from the "free" urine fractions of both female subjects, who were fed G.A. and MAG (Table I). Thus it is apparent that the carbohydrate moiety of MAG is hydrolyzed in the body to give the free G.A. as an excretion product. The infrared spectra of these urinary isolation products are the same as those of authentic G.A. and the fecal

TABLE II. Urine, Blood and Bile Levels of H^3-Labeled MAG and G.A.

Age	Sex	Adm. cpd.	% excreted in urine in 4 hr	% found in blood at 4 hr*	% excreted in bile in 4 hr
30	♂	1 mC MAG	.6	.09	
54	♂	*Idem*	.4	.0	
40	♂	"	.3	.2	.0
27	♂	2 mC G.A.	.2	1.2	
70	♀	*Idem*	.05	.4	.15

* Assumed blood vol = 4.2 l and HCT = 46%.

product shown in Fig. 1. These compounds isolated from urine do not, however, comprise the entire amount of radioactive material excreted in urine. Other radioactive "free" fractions are present which show different column chromatographic characteristics, so it is assumed that these represent other compounds

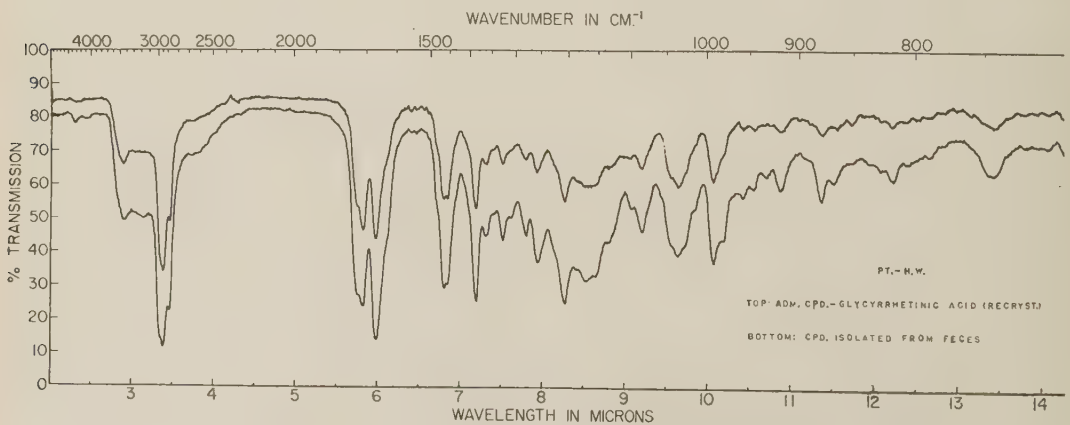

FIG. 1. Infra-red spectra in KBr of glycyrrhetinic acid, administered and isolated from feces.

not yet identified. In addition, identity of the conjugated material is unknown.

Discussion. Throughout the literature dealing with the physiological effects of G.A. and its glucuronide derivative MAG, it was necessary to give large oral doses each day for these effects to become manifest, and parenteral administration has not been feasible in view of their high degree of insolubility in aqueous media. From our results, it seems likely that the necessity for such large oral doses is due to the fact that MAG and G.A., when orally administered, are very poorly absorbed, so that oral doses of 3 to 4 g/day are probably necessary to result in very small circulating levels of parent compounds or their metabolites. Thus the actual circulating "effective amounts" of these compounds or their metabolites may be as small as those of many active steroids. If so, the problem may resolve itself into one of availability rather than one of physiological effectiveness. Variations among several G.A. samples in this regard, as reflected in greater or lesser degrees of absorption, may be related to the stero characteristics of the molecule, as discussed below. However, in view of the wide range of biological activities of MAG and G.A. demonstrated by many researchers, further studies on more efficient modes of administration and/or changing the molecule so that it may be more easily absorbed when given orally would seem to be indicated.

Unfortunately, when dealing with G.A. at the present time, one is faced with a lack of definite knowledge regarding the various stereoisomeric forms under which this compound may exist. And, since it can exist theoretically as many isomers, it is possible that these may possess different physiological as well as physical properties. This could explain the contradictory reports in the literature regarding effectiveness of G.A. in performing certain physiological functions, since the investigators often used material commercially sold by various sources as "glycyrrhetinic acid" or "Monoammonium Glycyrrhizinate," and failed to characterize their compounds physically. Thus the G.A. used in one laboratory may not have been at all the same as that used in another.

The G.A. used by us is likewise of uncertain stereoisomeric structure. Its infrared spectrum is identical with that of a commercial sample designated as beta G.A.§ as well as with that of an experimental sample of 18-beta G.A.‖ On the other hand, the specific rotation of our material is lower than that of the other 2 samples by 50 degrees, and its solubility in alcohol and chloroform is less. It is not 18-alpha G.A., since it is different in all properties, including infrared absorption, from a commercial sample of alpha G.A.§, which is probably the same as the 18-alpha compound of Beaton and Spring(11). Our compound has been purified by recrystallization, as well as by charcoal treatment of the Na salt, followed by precipitation of the free acid, and we have been unable to produce any significant change in either its infrared spectrum or specific rotation. The same procedures were carried out on both the commercial alpha and beta samples, again without change of characteristics. It may therefore be that our compound is beta oriented at the same position as are the others, but differs from them in some other position which does not cause a change in absorbance of infrared light but which affects the specific optical rotation. What this position might be we do not know.

Summary. Orally administered glycyrrhetinic acid and monoammonium glycyrrhizinate were poorly absorbed from the gastro-intestinal tract as indicated by blood, bile and urine levels of radioactivity. The bulk of fed materials are excreted directly in the feces, and, in the case of G.A., at least in part as unchanged compound. Small quantities of glycyrrhetinic acid have been isolated from urines of subjects fed each of above compounds. Many problems relating to stereoisomerism of G.A. remain to be clarified.

§ Generously supplied by Dr. R. R. Blumenthal, S. B. Penick & Co.

‖ Kindly donated by Dr. F. S. Spring, Royal Technical College, Glasgow, Scotland.

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Received July 13, 1959. P.S.E.B.M., 1959, v102.

Bone Sodium and Na^{22} Exchange: Relation to Water Content.* (25208)

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Reported values for sodium content of bone and for radiosodium exchangeability have shown considerable variation. Part of this variability is the result of age effects(1,2,3), for sodium content increases and exchangeability declines as animals become older. There still remains to be explained the variations noted among adult animals and men(1, 3,4,5). This report presents data on sodium content and radiosodium exchangeability for normal bone in a number of species, and demonstrates that the observed values fall into a regular pattern when allowances are made for variations in bone water content.

Materials and methods. The samples studied represent specimens of marrow-free bone obtained from normal animals. Human bone samples were obtained from subjects undergoing surgery, or from the autopsy room; none of the subjects suffered from disturbances of electrolyte metabolism. The human bone samples cover an age span from the 900 g premature infant to the adult, and those for the cat and rat from weanling to adult. The pigs were about 2 months old, the armadillo and fish of unknown age. The frogs ranged in weight from 3 to 85 g. A number of skeletal sites are represented: rib, skull, humerus and radius-ulna for the cat, pooled long bones of the rat, frog femur and tibia, pig and veal femur, armadillo rib, radius-ulna, and external bony plate, and rib, ilium, and long bones in man. Except for veal bone, all samples represent only the cortical portion. The human tooth samples† represent one each of a normal erupted, a normal unerupted, and a hypoplastic adult specimen. Enamel and dentin were carefully separated with the aid of a diamond grinding wheel. The teeth and veal bone had been frozen prior to analysis; all other samples of bone, including the goosefish (*Lophius americanus*),‡ represent fresh specimens. A number of bones were analyzed from this latter species, a marine teleost. Radiosodium^{22} was administered by the intravenous route to normal cats, and by the intraperitoneal route to normal rats, in a dose of 5 μc per 100 g body weight. Two hours later the animals were killed and samples of blood and bone removed for analysis. After removal of periosteum and marrow the bone samples were cut into small bits with an orthopedic rongeur, and dried *in vacuo* at 65°C. Fat was removed by ether extraction at room temperature, and the dry fat-free bone ground to a coarse powder in a mortar. Aliquots of this powder were then ashed in a muffle furnace at 525°C, and the ash taken up in 2N HCl. Sodium analyses were made either by a previously described column chromatographic technic(6) or by applying appropriate corrections to flame photometric readings of the ash

* Supported by grants from Atomic Energy Comm. with University of Rochester, and Nat. Inst. of Arthritis and Metab. Dis., P.H.S.

† Kindly provided by Dr. F. Brudevold, Eastman Dental Dispensary.

‡ Kindly provided by Dr. Roy P. Forster, Dartmouth College.

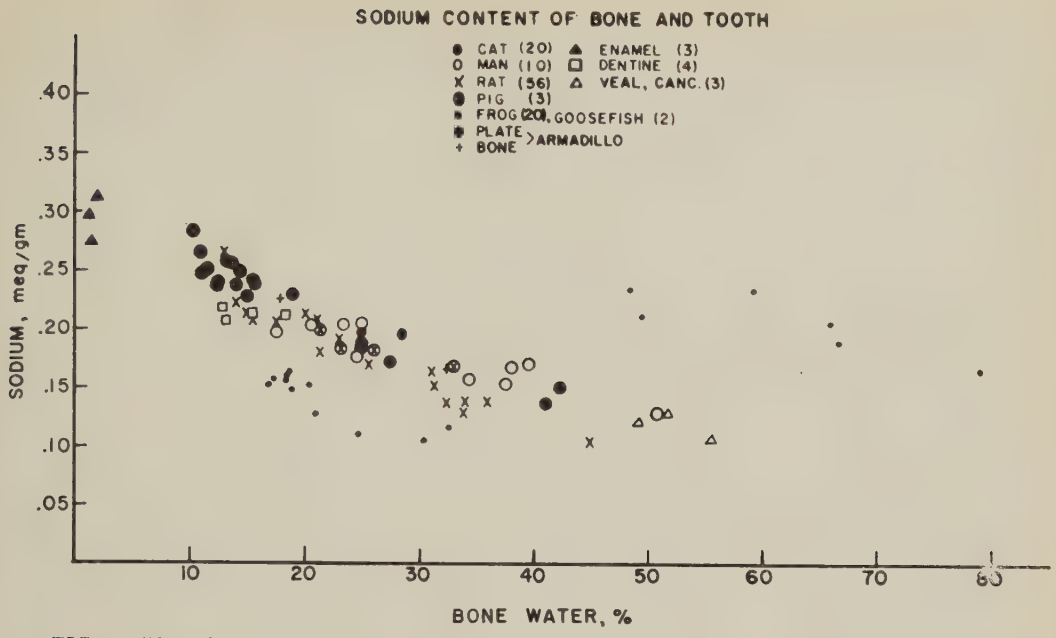

FIG. 1. Plot of sodium content against water content for various species. Number of animals indicated in parentheses. Values for the frog (•) lie below, and those for the goosefish (•) above the general curve. Samples of human bone depicted here all contained less than 5% fat.

solutions. Radiosodium[22] activity in bone ash solution and serum was determined in a scintillation counter, with appropriate correction for resolving time.

Results. Fig. 1 illustrates data for sodium content as a function of water content in the various species. In mammals there is an inverse curvilinear relationship between these two variables which is independent of species or skeletal site. It is of interest that such tissues as armadillo plate and human dentin conform to this general pattern, and that values for dental enamel are in fair agreement. Cancellous bone values can also be included.

It is evident that values for the 2 nonmammalian species do not conform to the general trend for mammals. These 2 species were deliberately chosen for analysis because they exhibit serum sodium concentrations far removed from the normal mammalian level of approximately 140 meq/l. In the 20 frogs we studied, where it is to be seen that bone sodium values fall distinctly below the general curve, the average serum sodium concentration was 110 meg/l. In the 2 goosefish, where bone sodium content is very high, serum sodium concentration averaged 194 meq/l. It would thus appear that bone sodium content reflects the concentration of this ion existing in extracellular fluid during bone formation. This contention is supported by previous studies(7) showing a reduction of bone sodium content in young rats fed a low sodium diet, with resultant hyponatremia.

One further discrepancy concerns a number of human bone samples containing large amounts (8-50% by weight) of ether-extractable material. These data, which have been omitted from Fig. 1, occupy positions well above the general curve for mammals when sodium and water contents are calculated on a fat-free basis. Samples containing 15% water, for example, have about 0.27 meq Na/g instead of the expected 0.24 meq/g; at the 30% water value these become 0.23 and 0.16 meq/g, respectively. Interestingly enough, the discrepancy is reduced somewhat when the calculations are made on a fat-containing basis.

In Fig. 2 radiosodium exchangeability is plotted against bone water content for the 2 species we have studied thus far. Here too there appears to be a relationship between these 2 variables, exchangeability mounting

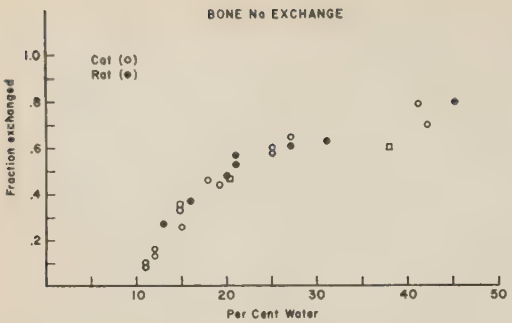

FIG. 2. Plot of radiosodium exchangeability against bone water content. Data for the cat represent individual samples, (cortical bone—○, cancellous—□; those for the rat averages of 3-11 animals.

as bone water increases. A similar phenomenon has been reported for radiophosphorus (8) and radiobromide(9). Fraction exchanged is calculated as the ratio bone: serum specific activity 2 hours following injection of radiosodium^{22}.

Discussion. The relationships depicted here provide a means of systematizing data on sodium content and radiosodium exchange in normal bone, and may prove useful in evaluating experiments involving a net change in bone composition. The conclusions of others (4,5) that bone sodium content varies from one skeletal site to another must be re-evaluated in the light of our findings; close inspection of the data from each of these reported studies also reveals an inverse relationship between sodium and water content. The previously reported effect of age(1,2,3) on bone sodium content may merely reflect age changes in water content. Of interest is the correspondence between tooth and bone and such unusual structures as armadillo plate, a fully calcified tissue. Cancellous bone exchange is in keeping with its water content. The observations of others(10) that radiosodium exchange in dentin exceeds that of enamel is also consistent with our findings.

The aberrant values noted for human bone samples of high fat content are difficult to interpret. A review of the individual data of Agna *et al.*(4) (Knowles, personal communication) reveals a similar phenomenon. Technical difficulties in removing such large amounts of fat may be at fault, though there is also the possibility that the supporting tissue associated with skeletal fat actually contributed a certain amount of extracellular electrolyte. This latter contention is supported by the finding of a higher chloride content in the high fat samples. For 10 samples containing less than 5% fat, the average Cl content was 0.029 meq/g fat-free wet weight, while that of the 11 high fat samples averaged 0.048 meq/g.

Finally, our data on frogs and fish can be offered in support of the hypothesis of Neuman and Neuman(11) that bone sodium content is a function of the extracellular fluid concentration of this ion.

Summary. 1) A study of marrow-free bone in a number of mammals reveals that sodium content is inversely related to water content. This relationship holds for a number of skeletal sites, and for dental structures. 2) Studies of 2 species with aberrant serum sodium values suggests that bone sodium content also varies, in direct fashion, with serum sodium content. 3) Radiosodium exchangeability of bone is also a function of bone water content.

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Received July 13, 1959. P.S.E.B.M., 1959, v102.

Stimulation of Irritable Tissues by Means of an Alternating Magnetic Field.* (25209)

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(Introduced by L. W. Roth)
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Observation of visual sensations not caused by light, by technicians working in the vicinity of large choke coils carrying strong alternating currents, was reported by Thompson (1). He was able to reproduce such an effect by exposing the head of human subjects to an extensive alternating magnetic field generated inside a large coil. This phenomenon ("phosphenes") has also been studied by others(2-5). The stimulation is evidently due to eddy currents induced in the tissues exposed to the alternating magnetic field. No attempt has been made in any of these investigations to localize the stimulus. The nearest approach to a search for local effects of an alternating magnetic field was an attempt by Magnusson and Stevens(4) to stimulate a cat's nerve by disposing it transversely in an alternating magnetic field. The result of this experiment was negative.

Methods. The magnetic fields used in the above-mentioned experiments were obtained with electromagnets excited with low frequency a.c. (below 90 cps) as well as with intermittent d.c. The observed light sensation was monochromatic (bluish white), flickering and strongest near the periphery of the retina.

To obtain a more nearly localized effect in our experiments, a bar electromagnet with a pyramidal pole tip, shown in Fig. 1, was constructed. Distribution of the axial field intensity component is shown in the same diagram. Currents of 2 frequencies have been used to energize this magnet, 60 cps and 1,000 cps. The maximum flux density obtained at the pole tip with the 60 cps source (a.c. line voltage) was 8,740 gauss (rms) and with the 1,000 cps source (motor-generator set), 2,260 gauss (rms). In spite of the lower flux density at 1,000 cps, the induced eddy currents are stronger as compared to those induced at 60 cps due to the proportionality of the induced e.m.f. to rate of change of the magnetic field.

Results. Observations of the phosphenes reported by previous authors have been verified at 60 cps. The flickering light sensation was strongest when the pole tip was held against the temporal area but could still be detected when the pole was placed against the occipital area. No other sensations or effects were observed in varying the location of the pole tip relative to different areas of the cortex.

At 1,000 cps a new effect was observed when the magnet pole was close to the temple. There was an intense sensation of nasal obstruction experienced by all subjects.

It proved possible to stimulate frog nerves intensely at 60 cps as well as at 1,000 cps by winding a nerve about the pole tip to form a closed one-turn loop. The pole tip was well insulated by a cap of acrylic plastic material ("Densoform"). A nerve-muscle preparation of a frog's sciatic nerve attached to the gastrocnemius muscle was used (Fig. 1). Intense tetanic contractions of the muscle were obtained whenever the magnet was excited. Fig. 2 shows a record of the muscle contraction with a simultaneous recording of the pattern of the stimulus. The latter recording was obtained by registration of the e.m.f. induced by the stimulating magnetic field in a loop surrounding the pole tip. The same effect has also been obtained in a substantially homogeneous 60 cps magnetic field of 6,000 gauss rms flux density. It also proved possible to stimulate contraction in excised frog muscles placed in a Petri dish and submerged in Ringer's solution. The Petri dish was placed into the gap of an electromagnet generating a 60 cps magnetic field in a 5 x 15 cm gap of 2.8 cm pole separation. The bottom of the Petri dish was parallel to the horizontally oriented pole faces. Tetanic contractions of freshly excised muscles were observed while

* This work supported by grant from Office of Naval Research.

the magnetic field was on. Stimulation of the irritable tissues by the eddy currents induced in the contents of the Petri dish showed a dependence on their position in the dish as well as on their orientation. The maximum effect was observed when the muscle was placed near the rim of the Petri dish and no effect was seen when the muscle was at the center. With the muscle placed near the rim, the contraction was strongest when the longitudinal axis of the muscle was oriented tangentially and least strong or absent for the radial orientation. These observations are in accordance with the anticipated distribution of eddy currents which flow along the rim of the Petri dish. The stimulating action of alternating magnetic fields depends on the induced eddy current density. It is reasonable to assume that an increase in intensity and, to a limited extent, in frequency of the alternating magnetic field used would lead to observation of effects which were not observed at the eddy current densities produced under the conditions described above.

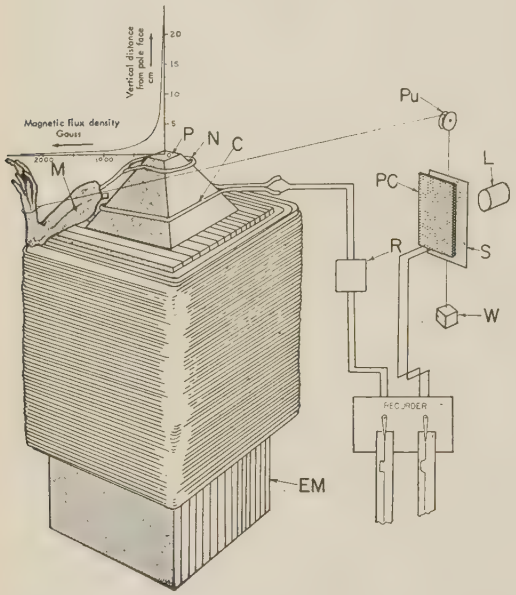

FIG. 1. EM: laminated bar electromagnet generating a non-homogeneous alternating magnetic field the axial component of which is represented in the diagram as a function of distance from the pole face P. N: nerve forming a closed loop in magnetic field. M: frog's gastrocnemius muscle. C: wire loop surrounding pole tip of the magnet. R: rectifier which rectifies the a.c. signal induced in loop C. The rectified signal is recorded by one of the 2 recorder channels. PC: photocell (International Rectifier Corp. type B-10) of the photoelectric device recording muscle contraction. L: light source. S: shutter which can be lifted by the string attached to frog leg. The shutter is actually flush with bottom of photocell PC. It is represented somewhat longer in the diagram to show the point of attachment of the string to which weight W is attached. When muscle contracts, shutter S and weight W are lifted thus exposing an increasing area of the photovoltaic cell PC to light from the source L. Voltage output of photocell is recorded by the second channel of the 2-channel recorder.

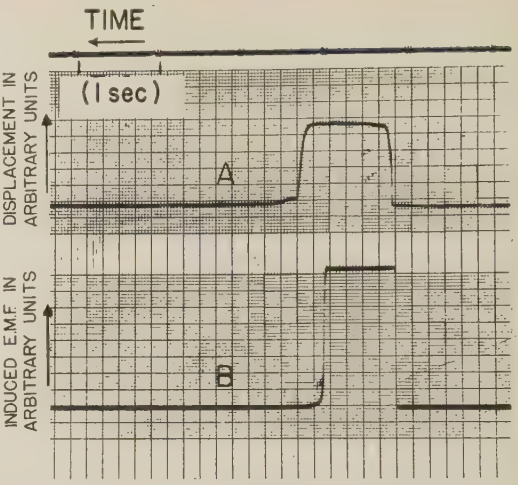

FIG. 2. Temporal relationship between stimulus and response in electromagnetic stimulation. A. Photoelectrically recorded movement of frog leg produced by contraction of the gastrocnemius muscle. Muscular contraction is evoked by stimulation of the nerve N of Fig. 1 through the current induced in it by the alternating magnetic field. B. The stimulus as a function of time in arbitrary units as represented by rectified and filtered e.m.f. induced in loop C of Fig. 1.

Summary. Stimulation of frog nerves and of excised frog muscles submerged in Ringer's solution has been obtained without the use of electrodes by means of sinusoidal alternating magnetic fields. This effect is due to eddy currents induced in conductive tissues and their surroundings. Visual and non-visual sensations have been induced in human subjects by non-homogeneous alternating magnetic fields adjacent to the brain.

The authors are indebted to Mr. R. T. Kado for valuable technical assistance.

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Received July 16, 1959. P.S.E.B.M., 1959, v102.

Cation Specificity of Thrombocyte Agglutinating Activity (TAg) of Canine Plasma.* (25210)

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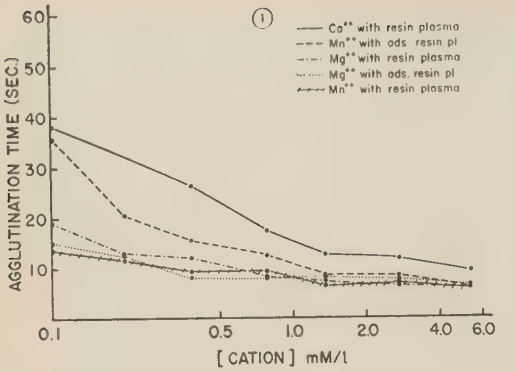
Canine plasma possesses the ability to agglutinate homologous and autologous platelets rapidly in the presence of Mg^{++} or Mn^{++}(1). This property of plasma has been termed thrombocyte agglutinating activity (TAg). TAg is contained in crude globulin fractions of plasma, but not in the albumin fractions. It is non-dialyzable and heat-labile. TAg appears to be separate from the plasma procoagulants, fibrinogen and antihemophilic factor, as well as from prothrombin and related $BaSO_4$-adsorbable factors. In this study TAg was tested for activity with 15 separate cations. The active ions were studied further to determine the relative amounts needed for prompt agglutination of platelets with TAg.

Methods. Details of the macroscopic platelet agglutination test for TAg have been described(1). The test is done in 2 stages. In the first or incubation stage, canine plasma is incubated for 30 min. with a cation chloride solution. In the second or agglutination stage, a standard suspension of washed canine platelets is added to the incubation mixture and time and degree of agglutination determined. Three types of canine plasma were used. *Resin plasma* was prepared by use of Dowex 50 resin columns(1). *Oxalate plasma* has been described(2). *EDTA plasma* was prepared by mixing 9 parts of whole dog blood with one part of stock EDTA solution(1), previously diluted one to 3.27 with normal saline. Plasma dialysis against 0.154 M NaCl was carried out with Visking casing (18 hr continuous agitation, 4°C). *Adsorbed plasmas* (resin, oxalate, or EDTA) were prepared by adding to each ml plasma 100 mg $BaSO_4$ (Merck) (contact time 30 min, 4°C) and repeating the procedure once. In the prothrombin time test with adsorbed plasmas no clot formed in 10 min. The chloride salts of various cations were used; all salts were Baker A.R., except $FeCl_3$ (Mallincrodt), $PbCl_2$ (Fisher), and $BaCl_2$ (Merck). Solutions were standardized by chloride analyses, and diluted to 0.108 M. For weaker solutions, serial 2-fold dilutions were made with 0.154 M NaCl. Cation concentration in the experiments is expressed as mM cation *added* per liter of plasma incubation mixture. *Platelet suspensions*(1) were prepared from EDTA canine plasma and contained $4 \cdot 10^5$ platelets/cmm. In several experiments, platelet preparation was modified by resuspending the platelets in solutions containing 0.5% bovine albumin (Armour). With this modification the platelet pellet could be resuspended rapidly, and platelet reactivity was unchanged.

Results. TAg and $BaSO_4$ adsorption of plasma. A series of tests were performed to determine the effect of $BaSO_4$ adsorption of plasma on platelet agglutination by TAg. Fig. 1 shows the results obtained with resin plasma before and after adsorption. With either Mg^{++} or Mn^{++}, rapid agglutination occurred, regardless of plasma adsorption or cation concentration used. Only with adsorbed plasma at the lower Mn^{++} concentrations were agglutination times less rapid than with non-adsorbed plasma. With Ca^{++}, on the other hand, no agglutination occurred with adsorbed plasma; with non-adsorbed plasma, clotting occurred in the incubation phase and the resultant serum caused moderately rapid agglutination.

* This investigation was supported in part by grant from Nat. Heart Inst., N.I.H., P.H.S.

† Post-Sophomore Research Fellow, N.I.H., P.H.S.



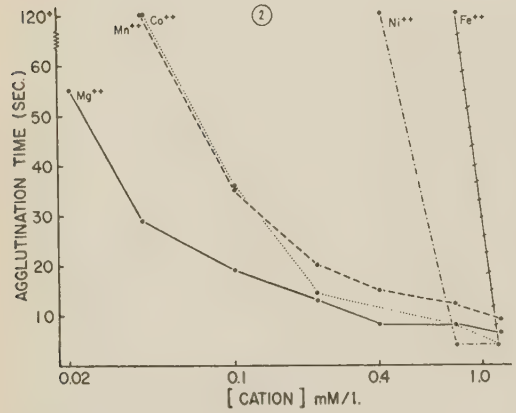


FIG. 1. Effect of $BaSO_4$ adsorption of plasma on TAg activity.

FIG. 2. Effect of varying cation concentration on TAg activity.

In subsequent tests adsorbed plasmas were used, thus avoiding clotting.

Cations and TAg. Resin, oxalate, EDTA, and dialyzed EDTA plasmas were each tested with 15 divalent or trivalent cations for TAg activity (Table I). With resin plasma, only 5 ions gave positive results: Mg^{++}, Mn^{++}, Fe^{++}, Co^{++}, and Ni^{++}. With plasmas prepared with the chelating agents, oxalate and EDTA, somewhat different results were obtained. With oxalate plasma, the same group of cations as well as Ca^{++} caused agglutination. With EDTA plasma, only Mg^{++}, Mn^{++}, and Ca^{++} were active. However, after EDTA was removed by dialysis, the plasma reacted with the same 5 ions as did resin plasma (last column, Table I), but not with Ca^{++}. With both oxalate and dialyzed EDTA plasmas, agglutination times were longer than with resin plasma.

Quantitative cation studies. The effect of varying cation concentration on TAg activity of resin plasma was next studied. Results obtained with ions possessing specific activity are shown in Fig. 2. Fe^{++} is required in relatively high concentration, while Mg^{++} is still active in comparatively low concentration. Ni^{++}, Co^{++} or Mn^{++} were somewhat less active than Mg^{++}. The other 10 ions were inactive in this dilution series.

Discussion. In this study of the effect of cations on TAg activity of plasma, a number of problems were encountered in obtaining conditions which would produce constant and reproducible results. Since $BaSO_4$ adsorption of plasma influenced TAg activity little, if at all (Fig. 1), the use of adsorbed plasma in the agglutination test was advantageous as it eliminated interference from fibrin formation in tests with Ca^{++} and Sr^{++}. Resin-treated plasma appeared to be more reliable than oxalate or EDTA plasma (Table I). In the presence of either oxalate or EDTA, false positive results were obtained with Ca^{++}. In the presence of EDTA, false negative results were obtained with Fe^{++}, Co^{++}, and Ni^{++}. After removal of the EDTA chelate by dialysis, the plasma gave qualitatively the same reactions with the different ions as did resin plasma. A number of factors appear to be responsible for the divergent results obtained with plasmas containing chelating agents. Included are plasma concentration of the added chelating agent, solubility of the chelate complex, and the stability constant or relative affinity of cation for chelating agent(3). These as well as other factors influence the availability of specific cations for platelet agglutination with TAg. Certain of the cations (see footnote, Table I) in high enough concentration caused both non-specific clumping of platelets in the absence of TAg and formation of a metal proteinate precipitate in plasma.

Of the cations which caused platelet agglutination with TAg, 4 of the 5—Mn^{++}, Fe^{++}, Co^{++}, and Ni^{++}—belong to the first transitional series of elements and have the atomic numbers, 25-28. The fifth ion, magnesium, is not a member of this series. Nevertheless, it was the most active of the ions tested (Fig. 2)—a concentration of only 1/2 - 1/40 as much as

TABLE I. Test of 15 Cations for Activity with TAg in Adsorbed Canine Plasma.

Cation	Atomic No.	[Cation] mM/l*	Adsorbed plasma tested: Resin Sec.	Resin Degree†	Oxalate Sec.	Oxalate Degree	EDTA Sec.	EDTA Degree	Dialyzed EDTA Sec.	Dialyzed EDTA Degree
Mg^{++}	12	21.6	5	4+	7	4+	7	4+	12	4+
		.78	10	4+	29	4+		Neg	‡	‡
Ca^{++}	20	21.6		Neg	16	2+	9	4+		Neg
		.78		"		Neg		Neg	‡	‡
Mn^{++}	25	21.6	6	4+	7	4+	5	4+	49	3-4+
		.78	8	4+	10	4+		Neg	‡	‡
Fe^{++}	26	.78	10	4+	17	3+		"	17	4+
Fe^{+++}	26	.78		Neg		Neg		"		Neg
Co^{++}	27	.78	5	4+	22	1+		"	15	4+
Ni^{++}	28	.78	6	4+	19	4+		"	31	4+
Cu^{++}	29	.78		Neg		Neg		"		Neg
Zn^{++}	30	.096		"		"		"		"
Sr^{++}	38	21.6		"		"		"		"
Cd^{++}	48	.78		"		"		"		"
Ba^{++}	56	21.6		"		"		"		"
La^{+++}	57	.21		"		"		"		"
Hg^{++}	80	.096		"		"		"		"
Pb^{++}	82	.096		"		"		"		"

* Greatest concentration of each cation was either 21.6 mM/l or that amount which did not cause a metal proteinate precipitate in the plasma during incubation stage of the test.

† Refers to size of platelet clumps graded macroscopically on scale of 1+ (about 5 platelets per aggregate) to 4+ (over 100 platelets/aggregate).

‡ Test not done.

Controls in which normal saline was substituted for plasma showed that the following cations, in ranges of concentration indicated, produced non-specific agglutination of washed platelets: Fe^{++}, 10.8-21.6 mM/l; Ni^{++}, Cu^{++}, and Pb^{++}, 5.36-21.6; Cd^{++} and La^{+++}, 2.72-21.6; Fe^{+++}, Zn^{++}, and Hg^{++}, 1.36-21.6. Controls with lesser concentrations and controls with the other cations did not cause non-specific agglutination.

that required for the other active ions was effective. Also, magnesium appears to be active in concentrations normally present in canine plasma(4). The other active ions, all "trace elements," appear to be required in concentrations higher than those normally present in plasma. It is of interest that the 2 ions, Ca^{++} and Sr^{++}, which are active in clotting were inactive with TAg in adsorbed resin plasma.

Summary. The effect of each of 15 divalent or trivalent cations on the thrombocyte agglutinating activity (TAg) of canine plasma was tested. Adsorption of plasma with $BaSO_4$ had little, if any, effect on TAg activity. In adsorbed resin-treated plasma TAg was active only in the presence of Mg^{++}, Mn^{++}, Fe^{++}, Co^{++}, or Ni^{++}, with Mg^{++} being effective in the lowest concentration. Seeming differences in cation specificity of TAg in plasmas containing the chelating agents, oxalate and EDTA, are discussed.

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Received July 20, 1959. P.S.E.B.M., 1959, v102.

Altered Reactivity to *Escherichia coli* Endotoxin of Mice Subjected to Sub-Lethal Tourniquet Treatment. (25211)

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One group of investigators has reported enhanced susceptibility of rabbits following hemorrhagic shock to the lethal action of the endotoxins of gram negative bacteria(1,2). The significance of this observation with regard to the pathogenesis of the irreversible stage of hemorrhagic shock remains controversial(2,3,4). The mechanisms through which susceptibility to bacterial endotoxin is increased are not completely known; and the extent to which this phenomenon operates in other animals and in shock produced by means other than hemorrhage has not been clearly defined. This study demonstrates that significantly less endotoxin was required to elicit a lethal end point in mice subjected to sub-lethal tourniquet treatment, and that prophylactic isotonic saline administration significantly reduced 24-hour mortality in mice which received endotoxin after tourniquet treatment.

Materials and methods. Webster strain white swiss mice of either sex, weighing 18-20 g, were employed throughout. The basic experimental design employed 4 groups of mice. *Group I* was subjected only to the standard tourniquet procedure (*vide infra*). *Group II* was given a single predetermined intravenous dose of *Escherichia coli* endotoxin contained in 0.5 ml physiological saline. *Group III* was subjected to the standard tourniquet procedure employed in Group I and was then given the same amount of endotoxin as Group II one hour after tourniquet removal. *Group IV* was given 4 ml of sterile physiological saline (at room temperature) intraperitoneally 30 min. after the standard tourniquet procedure. Thirty minutes following saline these animals were given *E. coli* endotoxin as in Group II. All mortality data were based on a 24-hour observation period. The *Escherichia coli* endotoxin preparation was extracted by the procedure of Boivin *et al.*(5) from cells grown in fluid cultures under forced aeration. The LD_{50} of a single intravenous dose of this material contained in 0.5 ml, determined by the method of Reed and Muench(6) from titration of serial 2-fold dilutions in normal mice employing 8 mice per dilution, was 117 μg. Sub-lethal tourniquet "shock" in mice, based on results of preliminary experiments, was induced by application of a tightly wound rubber band placed as high as possible on the left lower extremity. Constriction was released after 2 hours. The quantity of edema fluid in the injured extremity was determined by a previously reported bisection technic(7). Gain in weight of injured over normal leg permitted an estimate of the amount of extravasated fluid.

Results. Tourniquet application to one hind limb of a mouse for 2 hours was followed by marked local limb changes. Under these conditions, however, mortality over a subsequent 96-hour period following tourniquet removal was negligible, as indicated by 100% survival of 20 mice so treated in a preliminary experiment. The severity of the "state of shock" in mice subjected to tourniquet treatment could not be quantitated. However, the severity of the local damage could be gauged roughly by amount of edema, as judged by increase in limb weight over that of the opposite untreated limb. *Table I* records paired limb weights in a series of animals 24 hours after tourniquet removal and demonstrates that, on the average, tourniquet treated limbs weighed 0.32 g more than the untreated members, a gain in weight presumably caused by extravascular fluid accumulation.

Mice subjected to such sub-lethal tourniquet treatment, nevertheless, succumbed to lower doses of *E. coli* endotoxin than did nor-

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The author is indebted to Drs. Charles L. Wisseman, Jr., and Sheldon E. Greisman for encouragement, stimulation and evaluation throughout the study and to William Wood and Phyllis Auffarth for technical assistance.

TABLE I. Weight Increase in Bisected Hind Limbs 24 Hours after Tourniquet Removal in 10 Mice.*

	Limb wt (g)		
	Tourniquet side, left	Normal side, right	Difference (tourn. – norm.)
	.70	.47	.23
	.84	.53	.31
	.75	.46	.29
	1.00	.57	.43
	.84	.55	.29
	.78	.45	.33
	.82	.50	.32
	.94	.63	.31
	.95	.57	.38
	.90	.60	.30
Avg	.85	.53	.32

* Mean difference in wt of left hind limb over right hind limb of 10 normal mice was 0.05 g.

mal mice. This increased "susceptibility" was observed by 2 different experimental approaches. *Table II* shows, over a range of endotoxin dosages, increased death rate of tourniquet treated mice as compared to control mice. These results were observed regularly and are all statistically significant ($p < .04$). *Table III* demonstrates that only one-fourth as much endotoxin is required to elicit a 50% lethal end point in tourniquet treated mice as compared to controls. The results of Table III are reproducible and statistical analysis by the methods of Reed-Muench(6) and Pizzi(8) indicate $p < .01$.

Attempts were made to determine if the enhanced mortality of tourniquet treated mice receiving *E. coli* endotoxin could be prevented by prophylactic saline administration. Preliminary experiments indicated that 1-2 ml of physiological saline intraperitoneally did not prevent enhanced mortality. However, when 4 ml of physiological saline was given intraperitoneally 30 minutes after tourniquet removal and 30 minutes prior to 150 μg endotoxin, a protective effect was observed (Table II). These experiments were performed in triplicate with reproducible results and are statistically significant ($p < .01$). When 10 normal mice were pretreated with 4 ml. of physioligical saline 30 minutes prior to 150 μg endotoxin administration no protective effect was observed.

TABLE II. Mortality of Tourniquet Treated Mice Receiving *E. coli* Endotoxin.

		24 hr mortality after endotoxin dose*			
Group	Treatment	150 μg	100 μg	50 μg	12.5 μg
I	Tourniquet only	0/16†	0/16	0/16	0/16
II	Endotoxin "	8/16	5/16	4/16	2/16
III	Tourniquet and endotoxin	16/16	16/16	10/16	8/16
IV	Tourniquet saline‡ and endotoxin	9/16			

* Single intrav. dose of endotoxin 1 hr after removal of 2 hr tourniquet from 1 hind limb.

† Deaths/total.

‡ 4 cc of physiological saline given IP 30 min. after removal of 2 hr tourniquet from 1 hind limb.

Discussion. Significantly less endotoxin was required to elicit a lethal end point in tourniquet treated than in normal mice. The cause(s) of enhanced lethality in such mice, though unknown, might be associated with several factors, e.g.; (1) plasma loss, (2) passage of bacteria or their products across an intestinal membrane made increasingly permeable by ischemia, (3) release of toxic substances from the ischemic limb, (4) activation of the gas gangrene bacillus, (5) reticuloendothelial system injury.

TABLE III. LD_{50} of *E. coli* Endotoxin for Normal and Tourniquet Treated Mice.*

Group	LD_{50}† (μg)
Normal	117
Tourniquet treated	31

* Determined by single intrav. inj. of 2-fold endotoxin serial dilutions into groups of 8 mice/dilution.

† Calculated by method of Reed and Muench(6).

If mouse plasma volume be gauged from known mammalian plasma volumes(9), then roughly 25-35% of the original plasma volume of about 1 ml is lost into the tourniquet limb. Such mice experiencing a depletion of original plasma volume which in itself does not cause death, nevertheless, exhibit an enhanced lethality with *E. coli* endotoxin. However, if such mice are given large volumes of physiological saline (room temperature) intraperitoneally, no enhanced lethality is observed. Simple administration of large volumes of physiological saline has been shown (10) to reduce mortality in mice in which tourniquet treatment of both hind limbs would ordinarily lead to a fatal outcome. This amelioration of lethal effect acts presumably through a restoration of circulating blood vol-

ume. Indeed if lethal quantities of endotoxin diminish peripheral vessel reactivity to physiological regulators of vascular tone as described by Zweifach *et al.*(11) in an animal whose plasma volume is already reduced, a lethal outcome might result from cumulation of these two effects alone. However, since the protective effect of saline was obtained when administered early in the course of tourniquet shock and prior to endotoxin challenge, it is uncertain as to whether the protective effect was related to plasma volume replacement *per se,* and /or to inhibition of some train of events secondary to plasma volume depletion, such as increased absorption of bacterial endotoxin from the gastrointestinal tract.

Time sequence of introduction of bacterial endotoxin is probably important in susceptibility during shock. Zweifach and Thomas (12) have observed that rats treated with endotoxin *prior* to induction of non-lethal hemorrhagic or drum shock had a higher mortality than rats receiving endotoxin *after* such shock induction. It is emphasized that the 4-fold increase in susceptibility of tourniquet treated mice reported in this study is based on observations limited to a sequence of administration of endotoxin one hour *after* tourniquet removal.

The need for large amounts of saline in excess of estimated plasma loss to protect tourniquet treated mice receiving bacterial endotoxin may be attributed to the rapid loss of the administered saline into the injured area and adjacent tissue(13) and the slow peritoneal absorption. The observation that large amounts of saline failed to protect normal mice receiving endotoxin suggests that the saline does not act by simple endotoxin dilution and supports the concept that plasma volume loss constitutes the major factor in initiating the enhanced lethality of tourniquet treated mice receiving endotoxin.

Summary. Significantly less *E. coli* endotoxin (administered as single intravenous injection) was required to elicit a lethal end point in tourniquet treated mice than normal mice. Early intraperitoneal administration of large volumes of physiological saline significantly negated the effects of tourniquet treatment as regards lethal outcome with a given *E. coli* endotoxin dose. Such saline therapy failed to prevent death in normal animals given endotoxin. These observations plus the estimation of 25-35% original plasma volume loss to the traumatized area suggests that reduction of plasma volume is the major factor in initiating enhanced mortality of tourniquet treated mice receiving *E. coli* endotoxin.

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Received July 24, 1959. P.S.E.B.M., 1959, v102.

Griseofulvin and Human Spermatogenesis. (25212)

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Griseofulvin, a fermentation product of several species of Penicillium, has been shown to have marked inhibiting effects on morphogenesis of many fungi. Brian(1) has reported upon the *in vitro* activity of griseofulvin against 51 species of fungi and Campbell(2) has surveyed successful use of the antibiotic as an agricultural fungicide and its failure to produce toxicity in mammals under these conditions. As the therapeutic use of griseofulvin has shifted from the agricultural field to possible treatment of intractable fungal diseases in the human, legitimate emphasis has been placed upon the possible toxic effects of the drug. For example, recent studies in England(3) in which enormous doses of griseofulvin (up to 2 g/kg) were administered intraperitoneally to rats showed no overt reactions. However, histologic examination of the testicular tissue in these animals disclosed severe damage to germinal epithelium, and in many cases, complete necrosis of the testes. While we were aware that these effects were obtained in the rat only with rather overwhelming doses, we were interested in the possibility that, in lower concentrations, and in the human, a reversible inhibition of spermatogenesis could be produced. Our interest was stimulated further by preliminary clinical studies (4) which indicated that at dose levels of one to 2 g daily, griseofulvin was most effective against certain fungal infections in the human and did not produce unpleasant side effects.

Methods. We were fortunate in having access to a group of 14 men, inmates of an eastern penitentiary, who volunteered to take the drug for a period of 3 months at a level of 2 g daily. The latter dose, at present, is considered to be at least twice that necessary to produce remission of most of the fungal infections in the human which respond to this therapy. The 14 individuals were in the age group of 23-47 years (mean age 33), their physical condition was good, and they were living under similar rigid conditions of diet, exercise etc. Testicular biopsies were obtained from each during a short control period in which 2 semen specimens were examined, the second after 3 days of continence, to establish their level of semen quality. Thereafter, the griseofulvin was taken orally at the daily level of 2 g for a period of approximately 3 months. During this time, semen specimens were examined at weekly intervals, emphasis being placed upon ejaculate volume, sperm count, and sperm motility and morphology. A second testicular biopsy was obtained from 8 of the men at the end of the experimental period.

Results. Table I presents the mean counts/cc, total sperm counts and the mean readings of the spermatozoa morphology expressed as percentage of normal forms. The first control semen specimen examined was obtained after undetermined periods of continence, although in most of the 14 individuals this period was at least 2 weeks. The high mean sperm counts found in the first specimen may be accounted for on that basis. The second control semen specimen was obtained after only 3 days of continence (3 days after the first specimen) and should be considered as more representative than the first. Nevertheless, the mean sperm count figures for these 14 men both in the control specimens and thereafter during the entire course of the study, represent a population of high potential fertility. None of the 14 individuals in the study could be considered as an infertile individual and most were above average normal in all factors of semen quality. Motility readings are not recorded in the Table but without exception, they were good initially in controls and showed no serious deviation in any individual. Mean sperm counts showed a degree of fluctuation throughout the 12 week course of therapy but none that could be considered as outside the normal range of variation. Indeed, all aspects of semen quality, including sperm morphology, remained at a remarkably constant level. As would be expected from the

TABLE I. Average Semen Quality of 14 Men, at Weekly Intervals during 3 Month Period of Griseofulvin Intake.

	Semen specimen #	Ejaculate vol (cc)	Sperm count/cc — in millions —	Total sperm count	% normal forms
Control	1	3.42	228	780	79
"	2	2.84	185	527	78
	3	3.20	100	320	84
	4	3.30	175	577	84
	5	3.42	180	615	82
	6	2.91	134	389	81
	7	3.10	111	341	79
	8	3.10	117	363	83
	9	2.50	128	320	80
	10	3.48	130	452	81
	11	3.18	130	413	80
	12	2.74	123	337	79
	13	3.38	152	513	81

Semen specimens #3-13 produced at weekly intervals after intake of griseofulvin (2 g daily) was begun.

data on semen quality (Table I) the testicular biopsies did not show significant changes. Only 8 of the 14 individuals who had been on the griseofulvin for 3 months consented to the second biopsy. Attention was given to tubal size, patterns of spermatogenic activity, sperm production, condition of the peritublar connective tissue and number and functional appearance of the Leydig cells. In all these respects, the histologic condition of the testes appeared to be unaffected by treatment with griseofulvin. Both before and after the period of treatment the histologic picture of the biopsies obtained from the men in this series was, in each instance, within the recognized range of normality.

Summary and conclusions. Daily administration of 2 g of griseofulvin to 14 normal men for 3 months did not produce significant changes in semen quality or in histology of the testes of 8 of the individuals.

We are indebted to Dr. Shaffer of McNeil Labs., Philadelphia and Dr. Hawkins of Schering Corp. for the griseofulvin. We express our gratitude to Dr. A. Kligman of Pennsylvania for aid and to volunteers whose cooperation was so essential.

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Received July 27, 1959. P.S.E.B.M., 1959, v102.

Antibody Production in Neonatal Chickens Following Injection of Adult Cells Mixed with Antigen *in vitro.** (25213)

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Neonatal animals commonly exhibit an impaired immune response. They succumb easily to infection by pathogenic agents and do not respond, or react only poorly, to stimulation with non-living bacterial or protein antigens. Inability to produce circulating antibody following antigenic challenge appears to be a consistent feature among different species. Interspecies, interstrain, and individual variations, however, are characteristic of interactions between neonatal animal and transferred, viable lymphoid cells stimulated *in vivo* or *in vitro* with antigen(1-4). The variable reactivity of the chimera, *i.e.* the neonate containing transferred, stimulated, adult homologous lymphoid cells has been interpreted as representing a homograft reaction by Sterzl, Trnka, and co-workers(3,5), and the inadequacy or immaturity of the neonatal environment by Dixon and Weigle(1,8) and by Nossal(4). Our studies which confirm those of Trnka and Riha(2,3) demonstrate that antibody produc-

* Aided by grants from U.S.P.H.S., Minn. and Amer. Heart Assn.

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tion can be initiated in neonatal chickens by injecting adult spleen cells which have been stimulated *in vitro* with a bacterial antigen. Variation in response by individual recipient chicks, however, indicates that in this experimental model, utilizing non-inbred chickens, many factors may determine formation of antibody or lack thereof in the recipient host. Important considerations are the technical manipulations and supportive and/or repressive influences contributed by both graft and host, which may interact to different degrees.

Methods. White leghorn hens (Ghostley strain) were used as *donors for spleen cells.* Donor hens were anesthetized with sodium pentothal and bled by cardiac puncture. Immediately thereafter the abdomen was opened and the spleen removed aseptically. The excised spleen was then rinsed 2 times with Hank's solution containing penicillin (100 units/ml), streptomycin (100 μg/ml) and Nystatin (50 μg/ml)—RBSS, cut into 6 or 7 large fragments, and rinsed again with RBSS. Fragments were transferred to a sterile test tube covered with 2 ml of Eagle's medium containing antibiotics and heparin (2 units/ml), and homogenized by a loosely fitting pestle of a Potter-Elvehjem tissue grinder until a smooth uniform suspension was obtained. Microscopic examination of Wright-stained smears of suspensions at this stage of preparation revealed nearly all intact single cells with a few clumps. A viable cell count was made by using appropriate dilutions of a cell suspension in Hank's solution containing 0.5% Trypan Blue and recording the number of unstained white cells in a hemocytometer. Cells from one donor spleen were then divided into equal aliquots. One of the aliquots was injected intraperitoneally into one of a pair of recipient chicks as a control and the other aliquot was mixed with antigen prior to injection into the remaining member of the pair. A standardized suspension of *Brucella abortus* antigen (U.S. Dept. of Agriculture standardized diagnostic antigen, kindly supplied by Dr. Robert Lindorfer, School of Veterinary Medicine, Univ. of Minnesota) was made by counting cells in a Petrof-Hauser Counting Chamber under dark field illumination. A turbidity curve was plotted from dilutions of the standard suspension and the number of cells in any diluted suspension could be obtained from the curve after reading the absorbancy at 600 mμ. Dilutions of antigen were prepared to give a ratio of 2 bacterial cells to one viable spleen cell, and the resulting mixture was mixed by repeated aspiration and expulsions from a pipette. *Recipient chicks* of the same strain were obtained 12-36 hours after hatching and given injections as described in the experimental protocol below. *Six days after cell transfer the chicks were bled* by cardiac puncture and autopsies were performed on the animals from each experimental group. All blood for testing was allowed to stand at room temperature for one hour and overnight in the cold. After loosening the clot, the sample was centrifuged for 15 minutes and the serum removed. For *determination of agglutinin titers,* an initial 1:10 dilution of serum was made in phenolized, buffered saline (0.5% phenol in 0.01 M phosphate buffer containing 0.8 g NaCl/100 ml, pH 7.4) and thereafter serial 2-fold dilutions were made by adding 0.2 ml of the successive serum dilutions to tubes containing 0.2 ml of phenolized, buffered saline. To each tube, 0.2 ml of antigen (1:100 dilution of the original stock as supplied by Dept. of Agriculture) was added and incubation was carried out at 37°C with intermittent shaking for 24 hours. Immediately after removal from the water bath, tubes were examined in diffuse light with the aid of a concave, magnifying, reflector mirror. Titers were recorded as the last tube demonstrating agglutination after slight shaking.

Results. As controls agglutinin titers were measured in serum from the donor hen, normal chicks, chicks receiving Eagle's solution only, *Brucella abortus* antigen only, heated hen spleen cells (60°C for one hour) plus antigen, heated cells only, and viable cells only (Table I). In every case, control sera gave no agglutination ($<1:10$). All chicks were housed similarly; members of each group were distributed in each cage. Normal chicks were distributed among experimentals in order to detect any possible unfavorable factors in diet and housing. Mortality rates of chicks receiving spleen cells, antigen, or both may re-

TABLE I. Experimental Design for Study of Neonatal Chickens (12-36 Hours Post-Hatch) Receiving Transferred Homologous Adult Hen Spleen Cells by Intraperitoneal Injection.

Inoculum*	No. of recipient chicks	Mortality rate†
Viable cells + *B. abortus* antigen	35	9/35
Viable cells only	35	6/35
Heated cells (60°C for 1 hr) + *B. abortus* antigen	5	0/5
Heated cells only	5	1/5
B. abortus antigen only	20	7/20
Normal chicks‡	15	1/15
Eagle's only	4	0/4

* Each adult donor spleen was divided into 2 doses after counting the cells. Thus, chicks from each group receiving cells are part of a pair, one of whom received cells, the other, cells plus antigen.

† Mortality rate refers to chicks dying before 6th day after cell transfer.

‡ Normal chicks received neither cells nor antigen.

flect the toxicity of cells and/or of components of the antigen.

Antibody response in chicks receiving cells stimulated in vitro with antigen. Chicks receiving cells plus antigen responded in a variable manner (Table II). That the agglutinin titer was not directly related to the number of spleen cells can be seen from the table.

Gross pathologic changes in the chicks receiving viable cells plus antigen appeared to correlate well with antibody production. The most striking alterations consisted of splenomegaly, ectopic growths of splenic cells over the serosa of the intestine, hyperplasia of the visceral and parietal peritoneum, and occasional white, nodular overgrowths and capsular thickening near the lower border of the liver. Yellow gelatinous effusions in the subcutaneous tissue overlying the site of injection and occasionally over the entire peritoneal cavity were commonly noted. It should be emphasized that these gross pathologic changes were also found in chicks receiving cells only. Nevertheless, chicks which evidenced little or no pathologic change after receiving cells plus antigen did not produce any antibody which could be detected within the limits of the agglutination technic.

TABLE II. Agglutinin Titers in Chicks Receiving Intraperitoneal Injections of Adult Homologous Hen Spleen Cells Mixed with *B. abortus* Antigen *In Vitro*.

No. of cells $\times 10^8$	Reciprocal of titer	No. of cells $\times 10^8$	Reciprocal of titer
1.1	320	13.0	80
2.3	1280	13.0	2560
3.8	"	14.0	640
4.7	320	15.0	20
7.0	1280	15.0	320
8.0	<10	24.0	640
8.0	"	24.0	"
8.0	80	*	10
8.0	320	*	320
10.0	160	*	"
11.0	2560	*	640
12.0	"		

* Cells were not counted.

Antibody response in chicks receiving graded dilutions of cells and antigen. In an effort to define the limiting number of adult spleen cells necessary for transferring the antibody producing capacity in this system, decreasing 10-fold dilutions of cells were injected with antigen into neonatal chickens. From these experiments (Table III) it is apparent that higher titers can be obtained routinely by the intraperitoneal route only if more than 10^8 viable cells are introduced. Pathologic changes comparable to those described above were observed only in those chicks receiving 10^8-10^9 cells.

TABLE III. Agglutinin Titers in Chicks Receiving Intraperitoneal Injections of 10-Fold Dilutions of Adult Homologous Hen Spleen Cells Mixed with *B. abortus* Antigen.*

No. of adult spleen cells transferred	Reciprocal of agglutination titers†
10^9	40, 80, <10, D‡, D, <10, 1280, <10, <10, 160
10^8	<10, 320, 20, 160, <10, D, 320, <10, D, D
10^7	All <10
10^6	*Idem*
10^5	"

* Antigen was adjusted to maintain a ratio of 2 bacterial cells to one spleen cell.

† Titers represent individual chicks.

‡ D refers to chicks dying before 6th day after cell transfer when all remaining chicks were sacrificed.

Agglutinin titers of chicks receiving a constant number of in vitro stimulated adult spleen cells from the same donor. The variations in individual response noted in previous experiments utilizing different concentrations

TABLE IV. Variability of Response by Recipient Chicks to a Constant Dose of 10^9 Cells.*

Hen donor	Reciprocal of titer†
A	640, <10, <10, D, D‡
B	1280, 2560, D
C	<10, <10
D	<10, 320, 320, 640, D, <10
E	160, <10, D

* Each recipient received 10^9 cells from a single donor's spleen. Cells were stimulated *in vitro* with 2 bacterial cells/viable spleen cell.

† Titers refer to individual chicks.

‡ D refers to chicks dying before 6th day after cell transfer.

of transferred cells, prompted a study of the response by different chicks to a constant dose of cells and antigen from a single donor spleen. Variation among the individual recipients was found (Table IV). As in other experiments, the gross pathologic changes correlated with the antibody response. Absence of splenomegaly and associated alterations were consistently associated with little or no formation of antibody.

Discussion. In the experiments described above, transfer of spleen cells from normal, non-stimulated adult donor hens, mixed with antigen *in vitro,* resulted in antibody production by neonatal recipient chicks. Our results confirm the observations of Trnka and Riha(2,3) and attest further to the possibilities of using this experimental model to gain further information about the cellular mechanisms of antibody production, and associated aspects of transplantation immunity. In addition, antibody formation against added *Brucella abortus* antigen was noted only in recipient chicks which developed a characteristic pathologic picture, consisting primarily of splenomegaly. That this pathologic picture developed also in chicks receiving cells only indicates that antibody formation in the recipient chick may be correlated with a successful "take" by the transferred cells rather than by stimulation of the neonatal immunologic mechanisms or non-specific conditioning of the neonatal environment. When resistance in the recipient can be overcome by the transferred cells, the "graft versus host" reaction described by Simonsen(6,7) may develop. If this occurs, the transferred cells may react to both the antigenic stimulus provided by the host and any added antigens. Conversely, absence of antibody formation, splenomegaly, and associated changes may indicate either that successful transfer may not have been accomplished through simple mechanical failure or that the neonatal host may have overcome the immunological activity of the transferred cells by some form of resistance or lack of supportive nutritional factors. Experiments designed to clarify the function of transferred cells in formation of antibody by the neonatal recipients are in progress. Preliminary attempts to transfer the capacity to produce antibodies in response to added antigens through successive generations of chicks have been made. Results of these experiments, which are similar to Simonsen's transfer of the spleen enlarging factor(6), appear to support the observations outlined above and indicate that the pathologic alterations associated with antibody production are transmissible (unpublished).

In these studies no attempt has been made to compare quantitatively the response of newly hatched chicks and that of adult animals. Consequently, these data do not bear on the relative adequacy of the environment provided for transferred cells by the neonate and adult(8). However in these studies substantial antibody production has been achieved in newly hatched chicks. We feel that further study of this model may be useful in elucidating the mechanisms of antibody synthesis as well as the nature of the immunological deficit in immature animals.

Summary. Antibody production to *Brucella abortus* antigen was initiated in neonatal chicks by transfer of adult homologous hen spleen cells mixed with antigen *in vitro.* The studies indicate a wide variation in response and that a pathologic picture consisting primarily of splenomegaly is regularly associated with good agglutinin titers in the recipient.

The authors are indebted to James T. Prince for advice and help. We also acknowledge assistance of Marcia Marshall, recipient of Minn. Heart Assn. summer fellowship for high school students.

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Received July 28, 1959. P.S.E.B.M., 1959, v102.